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# **REMOVAL OF CRESOLS FROM SYNTHETIC WASTEWATER USING CRUDE SOYBEAN PEROXIDASE**

by

Kaushik Biswas

A thesis submitted to the  
College of Graduate Studies and Research through  
Civil and Environmental Engineering  
in partial fulfillment of the requirements for the  
Degree of Master of Applied Science  
at the University of Windsor

Windsor, Ontario, Canada  
1999

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## ABSTRACT

Soybean peroxidase (SBP) enzyme catalyzes the oxidation of various aqueous aromatic compounds in the presence of hydrogen peroxide to form polymers, which are readily precipitated from solution.

The enzyme used was extracted from soybean hulls and is called "crude soybean peroxidase". Experiments were conducted to develop a suitable protocol for extracting enzyme from soybean hulls and pertinent characteristics of the crude SBP enzyme were determined. These include the activity, thermostability, phenol content and the hydrogen peroxide demand of the crude enzyme. The results showed that the enzyme activity ranged from 50 to 80 units per gram of soybean hulls and it did not depend upon the volume of solvent added. The crude SBP was more thermostable (up to 80<sup>0</sup>C) than the commercially purified enzyme. The crude SBP had a high H<sub>2</sub>O<sub>2</sub> demand, which increased with an increase in enzyme activity. The phenol content of crude enzyme was directly proportional to the enzyme activity but was insignificant as compared to phenol concentrations in wastewaters.

Experiments were also conducted in batch reactors to investigate the efficiency of crude SBP in removing cresols from unbuffered synthetic wastewater. The optimum conditions to achieve at least 95 % removal of the cresols were determined for the following parameters: pH, SBP dose in the presence and absence of PEG (polyethylene glycol), hydrogen peroxide to substrate ratio and PEG dose. The results showed that crude SBP efficiently removed cresols from synthetic wastewater in the presence of hydrogen peroxide. The optimum pH ranged from 4.5 for p-cresol to 6.5 for o-cresol. An increase in the hydrogen peroxide to substrate ratio beyond the optimum did not change the removal efficiency. For each substrate, the optimum enzyme dose without PEG varied significantly, e.g. 0.15 U/mL for p-cresol to 0.25 U/mL for o-cresol. The studies showed that the addition of PEG caused no improvement in removal efficiency for o-cresol, decreased the removal efficiency significantly for p-cresol and increased the removal efficiency for m-cresol.

## **DEDICATION**

**This thesis is dedicated to my friends  
at the University of Windsor**

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## 1. INTRODUCTION

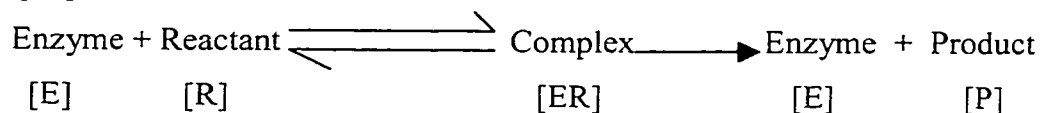
Cresols are isomeric substituted phenols with a methyl substituent at either ortho, meta or para position relative to the hydroxyl group. These compounds are contained in crude oil, coal tar, and flyash from coal and wood combustion (WHO, 1995). Cresols are considered to be toxic and have been classified as hazardous pollutants. There is clear evidence that during dermal or oral exposure in humans, high concentrations of cresols are corrosive, absorbed rapidly and produce severe toxicity that may result in death. The present treatment methods for removing aromatic compounds from wastewater, including biological treatment, chemical oxidation, adsorption on activated carbon and solvent extraction suffer from certain drawbacks. The introduction of an enzyme based technology by Klibanov *et al.* (1980) is expected to be a feasible alternative to the conventional treatment methods.

### 1.1 Enzyme Background

Traditionally, waste treatment processes have been evaluated in terms of reducing gross indicators of pollution such as biochemical oxygen demand (BOD), chemical oxygen demand (COD) or total organic carbon (TOC). Today, there is an increased emphasis on the removal of specific pollutants from waste mixtures and on the multimedia (air, land and water) effects of treatment processes. Existing waste treatment processes have limitations associated with the removal of specific chemicals to regulated levels. Enzymes represent one means by which selective removal of pollutants might be accomplished in waste removal. Enzymes catalyze chemical reactions that would otherwise be too slow at ambient temperatures to be of interest and therefore, can achieve chemical transformations that may be difficult to achieve efficiently with conventional chemical and biological treatment processes.

The use of enzymes in waste treatment applications was first proposed in the 1930s. However, the concept of using enzymes to destroy individual pollutants in waste mixtures was not developed until the 1970s. All enzymes are proteins, which are large molecules consisting of amino acid units joined in series. These biological catalysts increase the rate of the chemical reaction which is taking place (Palmer, 1981). The substrate of an enzyme is defined as the reactant of an enzyme-catalyzed reaction. The region, which contains the binding and catalytic sites, is termed the active site of the enzyme.

The kinetic feature that most distinguishes enzyme-catalyzed reactions from simple chemical reactions is that they show saturation. Enzymes, as catalysts promote chemical reactions but are not irreversibly modified themselves. Rate enhancements are attributed to a lowering of the activation energy of the reaction. In simple terms this is explained by the reactant physically binding to the enzyme at an “active site”, forming a metastable complex (Palmer, 1981). The activation energy for the breakdown of this complex is then considerably lower than that for the breakdown of the reactant alone. Starting from the simple concept the reaction scheme proposed is as follows:



The complex formation step is regarded as being reversible as the complex has a similar energy state to the enzyme-reactant mixture. The subsequent breakdown of the complex to give products will be exothermic and this stage may be effectively irreversible in many cases (Gacesa and Hubble, 1987).

The enzyme used in this study is a peroxidase which is extracted from soybean hulls called “crude soybean peroxidase” (SBP). Peroxidases oxidize reduced compounds in the presence of hydrogen peroxide. Several other peroxidases have been studied, with horseradish peroxidase (HRP) being the most extensively researched to date. Although the SBP enzyme has not been used in research as much as HRP, it is considered to be a suitable alternative to the other peroxidases. Previous studies (Al-Kassim *et al.*, 1995 and Caza *et al.*, 1999) have indicated that SBP is effective in removing several different phenolic compounds

from wastewater. This process produces a minimal amount of waste and is cost competitive with the other peroxidases that have been studied. In all of the previous studies with SBP, the commercially purified enzyme was used and no one had tried the “crude SBP”.

## **1.2 Objectives**

The objectives of this study were to:-

- extract enzyme from soybean hulls;
- determine pertinent characteristic properties of the enzyme and
- optimize the reaction parameters in unbuffered tap water, to achieve at least 95 % removal of cresols by using soybean peroxidase (SBP).

## **1.3 Scope**

The scope of this research included:

1. Establishment of a suitable experimental protocol for extracting enzymes from soybean hulls by optimizing parameters such as time of extraction and hulls to solvent ratio.
2. Determination of the characteristic properties of the crude SBP like the activity of the enzyme extracted, stability of the enzymes at different temperatures, amount of phenol present in the crude enzyme and hydrogen peroxide demand of the enzyme at different pH and enzyme doses.
3. Optimization of the removal of o-,m- and p-cresol with crude SBP with respect to: pH, SBP dose in the presence and absence of polyethylene glycol (PEG), molar ratio of hydrogen peroxide dose to substrate dose, and PEG dose.
4. Study of the effect of PEG on optimum enzyme dose.
5. Determination of the effect of co-precipitation with two different substrates on the optimum enzyme dose.
6. Comparison of the removal of cresol with commercially purified SBP enzyme and crude SBP.



## **2. LITERATURE REVIEW**

### **2.1 Cresols as pollutants**

Cresols are contained in crude oil and coal tar. Therefore, the dominant anthropogenic sources of cresols are accidental and process discharge during the manufacture, use, transport and storage of cresols or associated products of the coal tar and petroleum industries. Cresols are also produced during coal gasification, coal liquefaction and shale oil production. Low levels of cresols are present in the exhaust of vehicles powered with petroleum-based fuels, stack emissions from municipal waste incinerators, and emissions from the incineration of vegetable materials (WHO, 1995).

According to the Toxic Release Inventory (TRI) database, maintained by the US EPA, the total production-related cresol waste in USA for 1996 was more than 10 thousand tons. The total release was more than 900 tons. The TRI data may have under-estimated the actual release since only certain types of facilities were required to report (WHO, 1995).

There is clear evidence in humans that, during dermal or oral exposure, high concentrations of cresols are corrosive, absorbed rapidly and produce severe toxicity that may result in death (WHO, 1995).

Several populations have been identified that may be at special risk from cresol exposure. For instance, in persons with renal insufficiency, the renal clearance of phenol and p-cresol is impaired, leading to accumulation of cresol in the blood. Individuals with glucose-6-phosphate dehydrogenase (G6DP) deficiency may also have heightened sensitivity to the haematological effects of cresols (WHO, 1995).

Governmental regulations will require that a broad range of organic compounds in concentrations ranging from below  $\mu\text{g/litre}$  levels to 1000s of  $\text{mg/litre}$  must be detected and removed from aqueous streams even though they arrive at the detection/removal stage in a solution/suspension which can vary widely in other parameters such as temperature, pH, level of other solutes and

particulates. For example, in 1986 the Ontario Ministry of the Environment announced the Municipal Industrial Strategy for Abatement (MISA) Program whose objective is the virtual elimination of persistent toxic discharges into Ontario waterways. Future compliance with the discharge limits imposed by this and other similar programs will require the development of economically and technologically effective methods for reducing industrial and municipal discharges of targeted pollutants (Nicell *et al.*, 1993).

## **2.2 Cresols: Uses, Sources and Levels of exposure**

Cresols are used as solvents, disinfectants, and in the production of fragrances, antioxidants, dyes, pesticides, resins, and as wood preservatives. Cresols are contained in crude oil, coal tar, and fly ash from coal and wood combustion (WHO, 1995). The isomer o-cresol is used as a chemical intermediate for deodorizing and odour enhancing compounds and in pharmaceuticals. The compound p-cresol is mainly used in the formulation of antioxidants for lubricating oil and motor fuel, rubber, and polymers. The isomer m-cresol is used in the manufacture of explosives and in the production of herbicides and insecticides.

Exposure to cresol can occur through air, water or food. Surface water concentrations in the USA range from below the detection limit to 77 µg/L (STORET, 1993). Levels of 204 µg/L have been reported in a river polluted by industrial effluents in Japan. Concentrations as high as 2100 µg/L for o-cresol and 1200 µg/L for mixed m- and p-cresols have been detected in wastewaters (WHO, 1995).

## **2.3 Enzyme: Extraction**

A different purification procedure is usually necessary to isolate each enzyme, and there are many techniques that are available for the separation of proteins.

First and foremost, a suitable extract of the enzyme-containing tissue must be made. For many simple hydrolytic enzymes of microbial origin, the protein is secreted into the growth medium and removal of the cells by centrifugation is the only step that is required.

Extraction of enzymes from plant tissues presents a whole new set of problems and the comparatively small number that have been isolated is in part a reflection of the difficulties that are encountered. The cell wall of plants presents a formidable challenge. The forces needed to destroy the cell wall are so great that the desired enzymes are frequently denatured in the process. Furthermore, many plants contain phenolic compounds that are oxidized enzymically (polyphenol oxidases) in the presence of molecular oxygen to give products that can rapidly inactivate many enzymes. The pH, ionic strength and composition of the medium that is used for the extraction of an enzyme is important to the success of this and subsequent stages of purification (Gacesa and Hubble, 1987).

## **2.4 Enzyme: Stability**

Most enzymes have been shown to be moderately stable in the range 0-4°C. During storage, when catalytic activity is not important, this would represent the ideal temperature range. In some cases the presence of stabilizers, eg. glycols and sulphydryl compounds, have been shown to be highly beneficial. The presence of reactants or reactant analogues has also been shown to have a stabilizing effect on many enzymes (Gacesa and Hubble, 1987).

Intrinsically stable enzymes are generally found in organisms adapted to life in hostile environments. Although no clear principle has emerged, it appears that a degree of flexibility, allowing rapid renaturation, may be important. The greater stability of these enzymes must be balanced against the often higher cost of production when they are assessed for commercial applications (Gacesa and Hubble, 1987).

The stability of a given enzyme is a complex function of the environmental conditions used. It varies with pH, reactant concentrations and the

presence of destabilizing agents. Generally, the decay of enzyme activity is attributed to thermal effects, with the rate of decay being first order, reflecting the properties of the enzyme and its local environment (Cornish-Bowden, 1979).

For an enzyme to be suitable for commercial applications, it must be sufficiently stable for the purpose. In case of a reactor system, the stability can be quantified in terms of the profit made on the product formed during the lifetime of the enzyme catalyst. Whereas, for a sensor, the criterion would usually be based on the need for a linear response over an extended time period. This period would be determined by the cost of replacement and the run time of the process to be monitored. In addition to operational stability, ease and cost of storage must be considered. The rate of decay of enzyme activity,  $[E]$  with time,  $t$ , is given by:

$$\frac{d[E]}{dt} = -k_d[E] \quad 2.1$$

where  $[E]$  is enzyme activity and  $k_d$  is rate constant  
(Cornish-Bowden, 1979)

This equation can be integrated to give the active enzyme concentration at time  $t$  ( $[E']$ ):

$$[E'] = [E_0]\exp(-k_d \cdot t) \quad 2.2$$

where  $[E']$  = Active enzyme concentration at time  $t$

$[E_0]$  = Initial enzyme activity

So the decay constant,  $k_d$ , for the enzyme can be determined by plotting  $\ln([E']/[E_0])$  versus  $t$ .

The decay constant  $k_d$  is related to temperature according to the Arrhenius relationship, with the activation energy of the destabilization process being critical (Gacesa and Hubble, 1987). The presence of stabilizing compounds and the storage pH may change the activation energy and hence the necessary storage temperature.

The assessment of stability, both for storage and operation, is usually given in terms of half-life, i.e. the time taken for half of the enzyme to be lost, i.e. when

$$[E'] = \frac{[E_0]}{2}$$

Therefore,

$$\frac{[E_0]}{2} = [E_0] \exp(-k_d \cdot t)$$

or

$$t_{\frac{1}{2}} = \frac{0.693}{k_d} \quad 2.3$$

## 2.5 Peroxidase Background

Plant peroxidases possess a wide substrate specificity and, in the presence of hydrogen peroxide, can oxidize a large variety of aromatic compounds (Mc Eldoon *et al.*, 1995). These compounds are oxidized by hydrogen peroxide through an iron atom located at the catalytic site of the enzyme. The peroxidatic reaction mechanism is shown below (Dunford, 1991):



In this catalytic cycle, the native enzyme (E) is oxidized by hydrogen peroxide to an active intermediate referred to as Compound I ( $E_i$ ). Compound I accepts an aromatic compound such as phenol ( $AH_2$ ) into its active site. The aromatic

compound is oxidized resulting in the release of a free radical ( $\bullet\text{AH}$ ) back into the solution. The enzyme is now in the Compound II ( $\text{E}_{\text{ii}}$ ) state and oxidizes another aromatic compound, releasing a second free radical into solution. This final step returns the enzyme to its original state, thus completing the catalytic cycle.

The overall enzymatic reaction is as follows: -



The free radicals formed during this cycle diffuse from the active center of the enzyme into the solution where they combine to form dimers with reduced solubility that tend to precipitate out of the solution. The dimers which remain in solution, gets oxidized again resulting in the formation of a larger polymer, which in turn has further reduced solubility. This goes on until the polymer precipitates out of the solution. In the removal of phenol, the radicals formed are phenoxyl radicals that can couple with each other to generate various oligomers and polymers (Yu, J. *et al*, 1994).

Hewson and Dunford (1976) identified a major reaction product from HRP oxidation of p-cresol as Pummerer's ketone, a two ring compound proposed to result by reaction between the p-cresol radical and a second molecule of p-cresol. This reaction was suggested to be in competition with phenoxy radical coupling reactions (Hewson and Dunford, 1976).

Although Reactions (1) to (3) dominate in an aqueous mixture of enzyme, hydrogen peroxide and aromatic substrate, there are some side reactions that also occur which are believed to be responsible for inactivation and inhibition of the enzyme (Nicell *et al*, 1993). It has been reported by Arnao *et al.*, (1990) that once the enzyme is in the Compound II state, it can be oxidized by excess hydrogen peroxide to form Compound III ( $\text{E}_{\text{iii}}$ ) according to the following equation:



Compound III is equivalent to the oxy-ferro or superoxide-ferri-form of the enzyme. The return to the native enzyme is extremely slow, and therefore once in the Compound III form, the enzyme is sufficiently ineffective in carrying out the oxidation of aromatic substrates.

## **2.6 Wastewater Treatment**

The enzymatic method for treating phenolic compounds uses peroxidases in the presence of hydrogen peroxide to form phenoxyl radicals. These radicals couple to eventually form larger oligomers, which are practically insoluble in water and can be separated easily by filtration or sedimentation. Phenolic compounds such as cresols come from a variety of sources and this method has many advantages over conventional treatment processes.

### **2.6.1 Conventional Methods**

The selection of a waste treatment process involves the evaluation of many factors, including the technical feasibility with respect to one or more treatment objectives (permit requirements), economic feasibility, and the potential to generate residuals that require subsequent treatment or disposal. For wastes containing toxic components, treatment processes that destroy the toxic materials are preferred over methods that simply transfer these materials from one phase to another, for example, stripping of volatile organic compounds from wastewater into the atmosphere. Table 1.1 lists the current methods used for treating phenolic wastes. These conventional methods suffer from such serious drawbacks as incomplete removal, high cost, formation of hazardous by-products and applicability to a small concentration range.

Physical and chemical treatment processes, including activated carbon adsorption and the various oxidation processes developed in recent years are typically able to remove organic pollutants to low levels. However, most physiochemical treatment processes are not highly selective in terms of the range

of pollutants removed during treatment. Consequently, such processes are more economical for the treatment of dilute wastewaters and are often used as polishing steps.

**Table 2.1: Conventional Treatment Technologies for Phenolic Compounds  
(Nicell, 1991)**

|   |   |
|---|---|
| <b>Recovery Systems</b>                                 | Countercurrent extraction<br>Pulsed column extraction   |
| <b>Physical/<br/>Chemical<br/>Treatment<br/>Systems</b> | Chlorine oxidation<br>Chlorine dioxide oxidation (as sodium chloride)<br>Ozone oxidation<br>Hydrogen peroxide oxidation (Fenton's reagent)<br>Potassium permanganate oxidation<br>Incineration<br>Hydrocarbon stripping and combustion<br>Photocatalytic oxidation<br>Activated carbon adsorption<br>Landfilling<br>Coagulation |
| <b>Biological<br/>Treatment<br/>Systems</b>             | Bio-oxidation pond<br>Aerated lagoon<br>Stabilization pond<br>Oxidation ditch<br>Trickling filter<br>Activated sludge<br>Rotating biological contactors   |

In chemical oxidation, for example, oxidant dosages increase as the strength of the waste increases or as the required final concentration of the pollutant(s) of concern decreases. Therefore, chemical oxidation processes can



become prohibitively expensive for high strength wastes, even though the target pollutants might only be present in low concentrations.

Biological treatment, for example, is dependent on the health and activity of the microbial population. These microorganisms require sufficient food and oxygen, and stable environmental conditions, including pH and temperature, if they are to maintain optimum efficiency (Lanouette, 1977). Hence, with these shortcomings, alternative methods are desirable.

### **2.6.2 Use of enzymes**

Enzymes represent one means by which selective removal of pollutants might be accomplished in waste treatment. Such specificity allows enzymes to remove target pollutants selectively, while utilizing any required chemical reactants with very high stoichiometric efficiency. Enzyme specificity also precludes undesirable or unnecessary reactions, which would otherwise increase reactant consumption and correspondingly increase the cost of treatment. This is a great advantage over conventional chemical treatment processes. Therefore, enzymes have the potential to combine the advantages of selectivity with the simplicity, reliability and predictability of conventional chemical treatment systems.

The following potential advantages of an enzyme-based treatment over conventional biological treatment were noted by Nicell *et al.* (1993):

- application to a broad range of compounds;
- action on, or in the presence of, many substances which are toxic to microbes;
- operation at both high and low concentrations of contaminants;
- operation over wide temperature, pH and salinity ranges;
- no shock loading effects;
- no delays associated with acclimatization of biomass;
- reduction in sludge volume (no biomass generation);
- better defined system with simpler process control.

The same group also discussed the following potential advantages of an enzyme-based treatment over chemical/physical processes:

- operation under milder, less corrosive, conditions;
- operation in a catalytic manner;
- operation on trace level organic compounds and on organics not removed by existing chemical/physical processes;
- reduced consumption of oxidants;
- reduced amounts of adsorbent materials for disposal.

Klibanov *et al.* (1980) developed an enzymatic method for removing over 30 different phenols and anilines from wastewater using horseradish peroxidase (HRP). They noted certain reaction parameters, which were of significant importance, such as pH, hydrogen peroxide concentration and peroxidase concentration. Their results indicated that large amounts of HRP were required and, therefore, this method of treatment did not seem promising due to the high cost of the enzyme. Continued research showed that a great reduction in enzyme requirements could be achieved by using additives such as gelatin and polyethylene glycol (Nakamoto *et al.*, 1992; Wu Y. *et al.*, 1997) compared different additives in the removal of phenolic compounds and concluded that polyethylene glycol (PEG) was the best additive and that the amount of horseradish peroxidase required decreased significantly with an increase in PEG dose. Although HRP efficiently removed phenolic compounds from wastewater, it is unlikely ever to become a commodity suitable for this process due to the vagaries of root cultivation and processing (Taylor *et al.*, 1996).

## **2.7 Peroxidase in Wastewater Treatment**

The polymerization of aromatics in the presence of oxidoreductive enzymes, has recently been the focus of much attention due to its potential use for the decontamination of wastewaters. The enzymatic approach is particularly suitable for the treatment of wastewaters because during polymerization the

products form insoluble precipitates that can be removed easily from water by sedimentation or filtration.

Klibanov (1982), Klibanov & Morris (1981), Klibanov *et al.* (1980, 1983), Maloney *et al.* (1985, 1986) and Fiessinger *et al.* (1984) have demonstrated the effectiveness of using horseradish peroxidase for the polymerization and precipitation of substituted phenols and aromatic amines from wastewaters and drinking waters. These authors recognized the potential for the enzymatic treatment process and recommended the process for further development.

Although the enzymatic method has many advantages over conventional treatment technologies, it has a significant disadvantage: the relatively short catalytic lifetime of the enzyme. The short catalytic lifetime has been attributed to the inactivation of the peroxidase. Klibanov *et al.* (1983) has suggested that this inactivation occurs during the enzymatic reaction due to interactions of the phenoxyl radicals with the enzyme's active site. Therefore, this enzymatic method has not been considered to be a feasible option for application due to the extremely high cost of the enzyme.

Nakamoto and Machida (1991), on the other hand, have reported that enzyme inactivation is a result of the end-product polymer which adsorbs the enzyme and hinders the access of substrate to the enzyme's active site. They showed that the amount of enzyme could be greatly reduced by adding proteins or hydrophilic synthetic polymers, such as gelatin and polyethylene glycol (PEG). These additives inhibit the interactions between the enzyme and the phenolic polymers.

Wu J. *et al.* (1993) studied the effect of PEG on the minimum horseradish peroxidase (HRP) dose for 95% removal of phenol. They concluded that PEG protected the enzyme activity, and that 1/40 and 1/75 of the original amount of enzyme was required in the presence of PEG, for 1 and 10 mM phenol solutions, respectively. These results were confirmed by Wu Y. *et al.* (1997) where they compared different additives on the removal of phenolic compounds using HRP. The additives selected for the study were PEG, gelatin, and two polyelectrolytes, PERCOL LT24 (cationic) and PERCOL LT20 (non-ionic). Wu

Y. and co-workers concluded that polyethylene glycol was better than the other additives for several reasons. They found that excess PEG had no negative effect on the reaction whereas excess gelatin and the polyelectrolytes actually lowered the removal efficiency and also resulted in not forming particles. The effluent quality of the wastewater which was treated with PEG was better since at minimum PEG dose, there was little PEG left in solution; however, a considerable amount of gelatin remained in solution even at its minimum dose. Gelatin also produced more precipitate than PEG because the minimum gelatin doses were found to be higher than the minimum PEG doses. The results also indicated that the minimum HRP dose required for 95% removal of the aromatic compounds in the presence of PEG was much less than the original amount required without PEG for certain compounds (Wu, Y *et al.*, 1993).

The removal of phenols from a foundry wastewater using HRP was investigated by Cooper *et al.* (1995). These studies were conducted not only to optimize the operating conditions, but also to reduce costs. The economic feasibility of this process rested on reducing the cost of the enzyme. Two approaches were taken to minimize overall treatment costs: (1) evaluate the use of an additive to reduce enzyme requirements; (2) examine the potential of using a low purity enzyme to achieve equivalent removal at a lower cost. They found that PEG greatly reduced the cost associated with the enzyme. The use of PEG, a relatively inexpensive chemical, reduced the HRP requirements to 1/22 of the original enzyme requirements, which in turn greatly reduced the overall enzymatic treatment cost. Their results also showed that this process was capable of achieving 97-99% removal of total phenols from a foundry wastewater using either purified HRP or a crude HRP extract.

It has been suggested that easily removed aromatic compounds aid in the removal of other compounds with lower removal efficiencies (Klibanov *et al.*, 1980). These studies have shown that the removal efficiency of phenol increased in the presence of more easily removable compounds. Klibanov and co-workers have suggested two explanations, the first one being that phenol has a low reactivity towards peroxidase and is therefore poorly removed. Consequently, the

addition of more easily removed compounds increases the yield of free radicals which results in a higher formation of the polymeric products. The second explanation is that phenol is reactive towards peroxidase but the by-products have a low molecular mass and are soluble in water. The addition of compounds with higher removal efficiency results in the formation of a polymer with a higher molecular mass and therefore precipitates out of the solution.

## **2.8 Soybean peroxidase as an enzyme**

McEldoon *et al.*, (1995) have examined the substrate specificity of a peroxidase isolated from soybean hulls (SBP). In their study, they found that plant peroxidases, such as SBP and HRP, possess a wide substrate specificity that can be dramatically broadened at low pH values to include nonphenolic compounds. SBP follows a ping-pong, bi-bi catalytic reaction mechanism. It is found to be highly stable at pH 2.4 and also thermostable under acidic conditions.

Caza *et al.*, (1999), have shown that soybean peroxidase is a viable alternative to other enzymes that have previously been studied. The results of the study have demonstrated the applicability of using SBP for treating synthetic phenolic wastewater. The reaction parameters, optimized to achieve a removal efficiency of at least 95%, were pH, SBP dose with and without PEG,  $[H_2O_2]/[Substrate]$  and PEG dose. The study recommended the necessity of investigating the potential of using soybean hulls instead of purchasing the enzyme from a chemical manufacturer. After the enzyme has been extracted from the hulls, they could then be used as animal feed. This would greatly reduce the amount of solid waste that is produced and in turn would also reduce costs. Similarly, some researchers have also suggested using crude HRP (Cooper *et al.*, 1996; Dec *et al.*, 1994; Klibanov, 1982) to reduce treatment costs. In contrast to crude SBP, the amount of solid waste produced with the use of crude HRP is much greater. Another advantage of using soybeans is that they are more readily available than horseradishes.

### **3. MATERIALS AND METHODS**

The entire work was divided into two sections A and B. Section A involved the extraction and characterization of crude soybean peroxidase enzyme and Section B involved the polymerization and removal of substrates.

#### **3A. Extraction and characterization of crude soybean peroxidase enzyme**

##### **3A.1 Materials**

Soybean hulls were obtained from ADM-Agri Industries Limited, Windsor, from two different batches (called A & B). A was 5 years older than B and while the former was stored at 4<sup>0</sup>C in dark the latter was obtained fresh. The activity of the enzyme extracted from the hulls, was determined by using the enzyme activity assay described in Appendix A (Wu, 1993). A unit of activity is defined as the number of micromoles of hydrogen peroxide converted per minute at pH 7.4 and temperature 25<sup>0</sup>C. The hulls were stored at room temperature, while the enzyme extract was stored at 4<sup>0</sup>C.

Hydrogen peroxide (30% by mass over volume) was purchased from BDH Inc., Toronto, Ontario and was stored in a refrigerator at 4<sup>0</sup>C. The diluted hydrogen peroxide solutions used in the analysis were prepared daily.

Whatman's glass microfiber filters of 42.5 mm diameter (934-AH) was purchased from Whatman Inc. Clifton, NJ 07014, USA. Also, millipore filters of Type HA and size 0.45µM were purchased from Millipore Corporation, Bedford, MA 01730.

##### **3A.2 Analytical Equipment**

The rate of colour absorbance for enzyme activity was measured using a Hewlett Packard Diode Array Spectrophotometer Model 8452A (with a wavelength range of 190 to 820 nm and 2nm resolution), which was controlled by a Hewlett Packard Vectra ES/12 computer. Polystyrene disposable semi-micro

cuvettes were used. They were purchased from Bio-Rad Laboratories, Hercules, CA. An IEC Centrifuge, supplied by International Equipment Company, USA; was used to separate suspended particles. It was operated at 4000 r.p.m. for 5 min.

An Expandable Ion Analyzer EA 940, manufactured by Orion Research, was used to measure the pH of the samples. Standard buffer solutions of pH 4.0, 7.0 and 10.0 were purchased from BDH Inc., Toronto, Ontario.

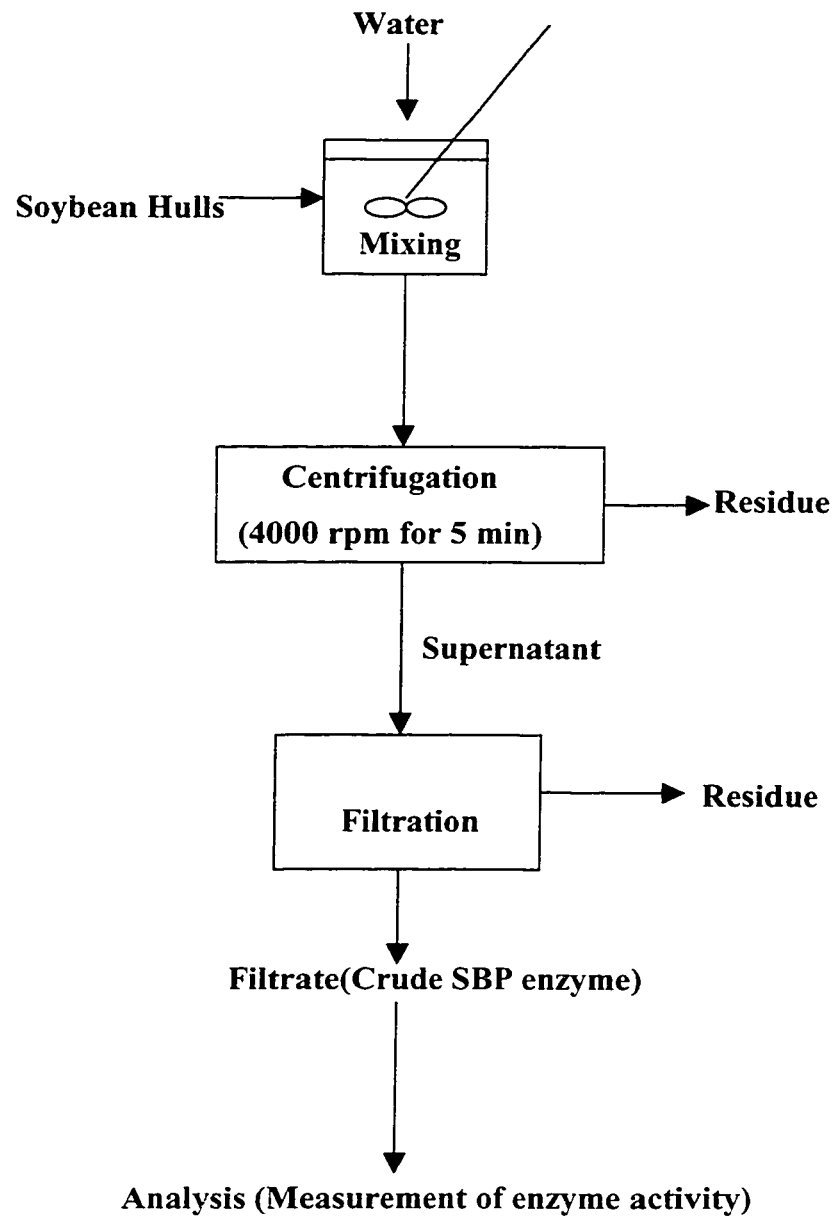
### **3A.3 Experimental Procedure**

#### **3A.3.1 Extraction of soybean peroxidase from soybean hulls**

The experiments were conducted at room temperature of approximately 22°C. The study was designed to develop a suitable experimental protocol for extraction of soybean peroxidase (SBP) from soybean hulls. Soybean hulls was obtained in two different batches (A & B) of hulls. Tap water and phosphate buffer solution (0.1 M, pH=7.4) [Gomori, 1955] were used as solvents in order to extract the enzyme from the hulls. Buffer solution was used so that the pH remained stable during extraction.

10 g of soybean hulls was added to tap water and buffer solution separately and mixed thoroughly with magnetic stirrer. After mixing, the hulls were separated from the solution, first by centrifugation and then by filtration. Irrespective of the amount of solvent used about 50 mL of the solvent could not be recovered in the extraction process. Then the activity of the extracted enzyme solution was determined. This enzyme solution extracted from soybean hulls is termed as “crude soybean peroxidase”. The parameters that were varied in order to determine the optimum conditions were time of extraction and hulls to solvent ratio.

Initially, after the mixing of hulls and solvent, separation of the extracted solution from the hulls was tried by filtration alone. But, it was found that the hulls clogged the filter paper and prevented filtration to take place. Consequently, centrifugation was applied before filtration and during this process a significant



**Figure 3A.3.1: Flowchart for the extraction of SBP from soybean hulls using tapwater**



amount of the hulls was separated from the extracted solution. Thus the supernatant, obtained after centrifugation could easily be filtered and analyzed.

It was also observed that by using a millipore filter of Type HA and size 0.45 $\mu$ M instead of a glass microfiber filter, the total units of enzyme extracted were reduced by several fold. Therefore, glass microfiber filter was used for filtration purpose. Figure 3A.3.1 shows the procedure adopted for enzyme extraction using tap water. The extraction time was varied between 0.5 to 5 h.

### **3A.3.2 Temperature stability of soybean peroxidase**

The experiments were conducted in order to determine the changes in the activities of commercially purified SBP and crude SBP (A & B) due to exposure to high temperatures for different durations of time. A small volume (approx. 5mL) of the enzyme solution with a known activity (measured previously) was taken in different test tubes and dipped in water bath maintained within particular temperature ranges. After heating the enzyme solutions for a certain time, they were instantly taken out of the water bath and cooled to room temperature by keeping in a refrigerator. The activities of the enzyme solution were then determined and compared with the ones measured before heating. The parameters that were varied in order to study the enzyme characteristics were the temperature ranges and the time of heating. If the enzyme activity did not change appreciably due to high temperatures, the enzyme was considered to be “stable” within that temperature range.

### **3A.3.3 Hydrogen peroxide demand of crude SBP enzyme at different enzyme doses and pH**

Batch experiments were conducted at room temperature, approximately 22<sup>0</sup>C, to determine the hydrogen peroxide consumed by crude SBP at different pH values when mixed for 3 h. The batch containers were glass vials, which contained 30 mL of a mixture of SBP, hydrogen peroxide and tap water. The tap

water was boiled to remove the chlorine and then cooled down. The components of the sample mixture were added in the following order: SBP, tap water and hydrogen peroxide. For each experiment, there was one control vial, which contained components except SBP. At the end of stirring for 3 h, the remaining hydrogen peroxide was found with the help of the  $\text{H}_2\text{O}_2$  assay procedure given in Appendix C. For determination of the pH effect, the pH was adjusted before the addition of hydrogen peroxide to the desired value. The pH after the mixing process was also determined in order to check whether or not there was a change in pH due to the mixing process.

Since crude enzyme solution may contain other constituents beside the enzyme, another experiment was run by using the enzyme solution in which enzyme had been denatured by boiling for 30 minutes. The activity was measured both before and after boiling the enzyme solution and it was found that the enzyme solution retained less than 1% of the enzyme activity after boiling. Therefore, it was assumed that the enzyme was denatured.

Thus, the hydrogen peroxide demand was determined both for crude SBP enzyme and killed crude SBP enzyme at different enzyme doses and pH.

#### **3A.3.4 Phenol content of crude SBP enzyme**

Crude enzyme may contain phenolic compounds and this might interfere with the analysis during the use of crude enzyme for the removal of phenolic compounds. So it was decided to determine if the phenolics present in the crude enzyme were significantly large to influence the analysis of phenolic compounds in synthetic wastewater.

The phenol present in the crude SBP was determined by using the enzyme as the sample against the standard calibration curve of phenol given in Appendix B.

### **3A.4 Analytical Methods**

#### **3A.4.1 Phenol Concentration Assay**

The concentration of the phenol was determined by a colorimetric method. The concentrations of the substrates are expressed as molar quantities, where one millimolar is equal to 94 mg/L of phenol. The colorimetric method used 4-aminoantipyrine (AAP) and ferricyanide as colour generating substances when combined with phenolic compounds. The colour generated at a peak wavelength of 510 nm was directly proportional to the concentration of the aromatic compound. The assay mixture in the plastic cuvette consisted of 100  $\mu$ L of ferricyanide solution, 100  $\mu$ L of AAP solution, 200 to 800  $\mu$ L of aromatic sample, and deionized water to bring the total volume to 1000  $\mu$ L. A detailed procedure for the colorimetric method can be found in Appendix B (Caza *et al.*, 1999).

#### **3A.4.2 Peroxidase Activity Assay**

The enzyme activity was measured with peroxidase activity assay procedure which used phenol, 4-AAP and  $\text{H}_2\text{O}_2$ . This method provided all reagents in excess except for enzyme in order to ensure that the initial rate of reaction was directly proportional to the amount of SBP present. Enzyme activity is defined as the number of micromoles of hydrogen peroxide converted per minute at pH 7.4 and 25<sup>0</sup>C. The assay mixture consisted of 100  $\mu$ L of 100 mM phenol, 250  $\mu$ L of 9.6 mM 4- AAP, 100  $\mu$ L of 2 mM  $\text{H}_2\text{O}_2$ , 500  $\mu$ L of 100 mM phosphate buffer (pH 7.4), and 50  $\mu$ L of enzyme sample. Immediately after the addition of the enzyme, the cuvette was shaken and the change in rate of absorbance with time was monitored at a peak wavelength of 510 nm which was converted to a rate of change in  $\text{H}_2\text{O}_2$  concentration based on an extinction coefficient of 6000  $\text{M}^{-1} \text{cm}^{-1}$ . A detailed description of this method can be found in Appendix A (Wu *et al.*, 1997).

### **3A.4.3 Hydrogen Peroxide Assay**

A colorimetric assay has been developed for the measurement of hydrogen Peroxide ( $\text{H}_2\text{O}_2$ ) concentration using ARP as catalyst and AAP as colour generating substances. This assay procedure was set up so that the only substance present in limiting quantities was hydrogen peroxide. Therefore, the intensity of colour formed at 510 nm was directly proportional to the amount of peroxide originally present in the sample. The assay sample volume was kept at 1 mL. The peroxide concentration in the cuvette did not exceed 50  $\mu\text{M}$ . The maximum colour formation at the peak wavelength was recorded. From the calibration curve the amount of peroxide that was present in the cuvette was determined. If the value exceeded 50  $\mu\text{M}$ , then the process was repeated but using smaller sample volume and the difference was made up with buffered water so that the assay volume was always equal to 1 mL. Once a satisfactory cuvette concentration was achieved, the amount of peroxide present in the original sample was found by back-calculation.

### **3A.5 Sources of Error**

In any experiment, several errors may affect the reliability of the results. Systematic errors are due to analytical techniques and instruments, whereas random (or human) errors are due to personal factors. Calibration curves were done several times and compared to verify the accuracy. A set of experiments, the hydrogen peroxide demand of crude SBP at doses of 0.1, 0.4 and 0.8 U/mL, was repeated 3 times and the results were compared to determine the reliability of the experimental results.

### **3B. Polymerization and removal of substrates**

#### **3B.1 Materials**

Additional materials required are described below:

Catalase (EC 1.11.1.6, 15000 units/mg solid) and polyethylene glycol (with an average molecular mass of 3350 g/gmole) were purchased from Sigma Chemical Co., St. Louis. MO. One unit of catalase decomposes one micromole of hydrogen peroxide per minute at pH 7.0 and temperature 25<sup>0</sup>C. An aqueous stock solution of catalase was stored at 4<sup>0</sup>C.

O-cresol, m-cresol, p-cresol and phenol with a purity of 99% or greater, were supplied by Aldrich Chemical Co., Milwaukee, WI. Stock solutions were stored at 4<sup>0</sup>C.

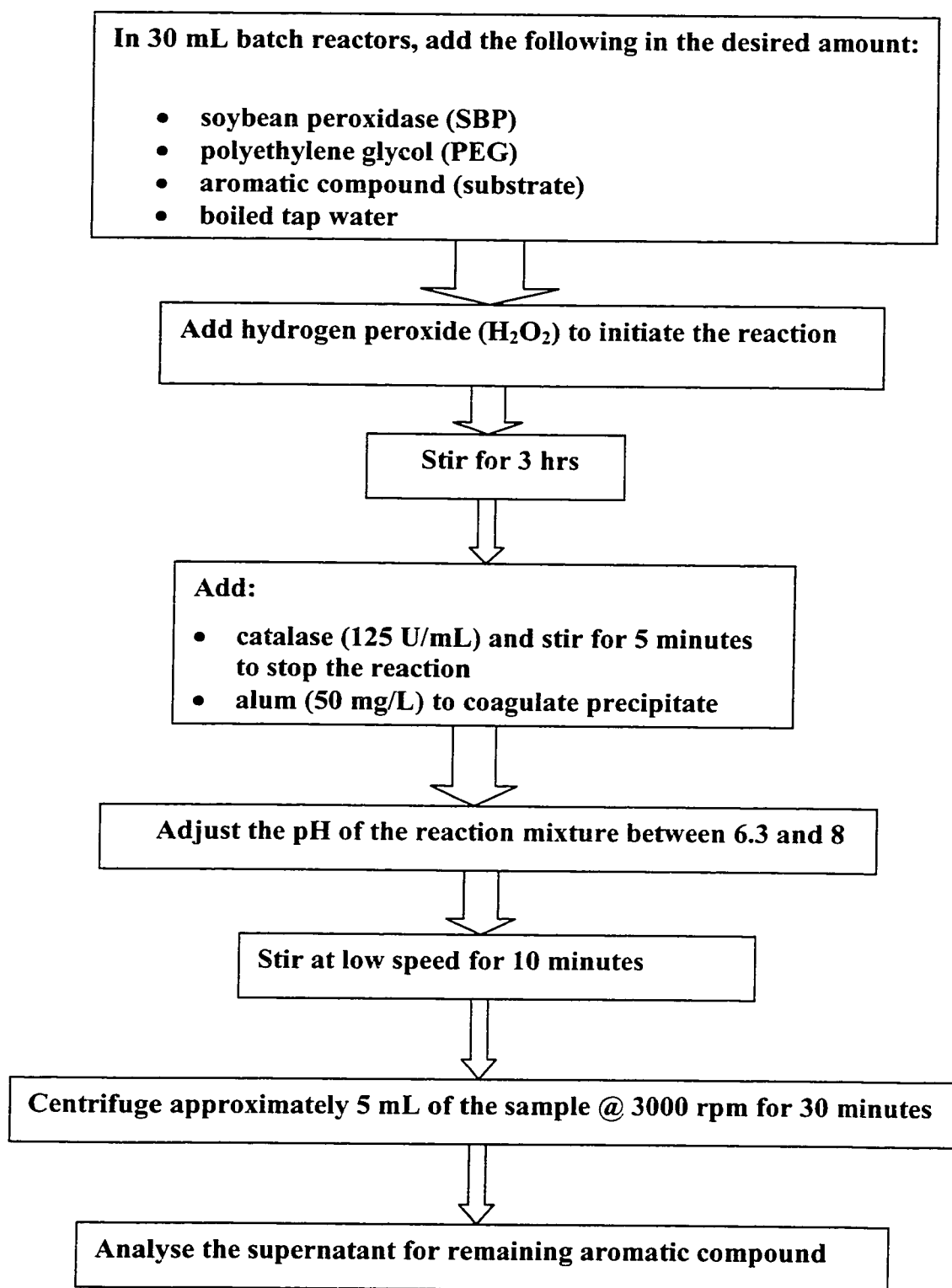
#### **3B.2 Analytical Equipment**

In addition to the analytical equipment described previously, quartz cells with a 10 mm path length were purchased from Hellma (Canada) Limited, Concord, Ontario.

#### **3B.3 Experimental Procedure**

Batch experiments were conducted at room temperature, approximately 22<sup>0</sup>C. This study was designed to achieve at least 95% removal of the aromatic substrate by optimizing the following parameters: pH, PEG dose, SBP dose both with and without PEG, and H<sub>2</sub>O<sub>2</sub> dose.

The batch reactors were glass vials, which contained 30 mL of a mixture of aromatic substrate, H<sub>2</sub>O<sub>2</sub>, PEG, crude SBP enzyme and tap water. The tap water was boiled and cooled down to room temperature in order to remove the chlorine. The components of the sample mixture were added in the following order: aromatic substrate, PEG, crude SBP enzyme and tap water. Reactions were



**Figure 3B.3.1: Flow Chart for Experimental Procedure**

initiated by adding a known amount of  $\text{H}_2\text{O}_2$  to each reactor. For each experiment, there was one control reactor, which contained all other components except  $\text{H}_2\text{O}_2$ . The control reactor was used to measure the initial amount of the aromatic substrate present in the sample mixture. All of the reactors, including the control reactor, were stirred vigorously for three hours using a magnetic stirrer and teflon coated stir bars. At the end of the reaction period, catalase was added to a final concentration of 125 U/mL to stop the reaction and alum was added to a final level of 50 mg/L to the mixture and stirred. The pH of each sample was adjusted to between 6.3 to 7.5 and then stirred again at low speed to allow for the formation of the floc. Approximately 5 mL of each sample was centrifuged, after which the supernatant was analyzed in duplicates for remaining substrate by the colorimetric method. The following four sections and Figure 3B.3.1 describe the procedure used to optimize all of the reaction parameters.

### **3B.3.1 pH**

The first parameter that was optimized was pH. The reaction mixture consisted of tap water, aromatic substrate, PEG and SBP enzyme. The substrate concentration was 1mM for all of the different cresols that were tested. The SBP dose used in these experiments was determined from previous studies and the PEG dose in each reactor was 400 mg/L. The pH was then adjusted within the range of 3.00 to 10.00 using concentrated HCl or NaOH.

### **3B.3.2 Peroxidase Dose**

The optimum SBP dose was determined both in the presence and in the absence of PEG. In one set of experiments, an excess of PEG, 400 mg/L, was added to the reaction mixture, whereas in the other set of experiments, no PEG was added. These tests were conducted at the optimum pH values determined in the previous set of experiments. Peroxidase was added in pre-determined amounts

in order to determine the minimum SBP dose required to remove 95% of the initial substrate concentration.

### **3B.3.3 Hydrogen Peroxide Dose**

The next parameter to be optimized was the  $\text{H}_2\text{O}_2$  concentration. The amount of hydrogen peroxide, which was added to the reactor, is expressed as a ratio of millimolar  $\text{H}_2\text{O}_2$  to millimolar aromatic compound ( $[\text{H}_2\text{O}_2]/[\text{Substrate}]$ ). During this set of experiments, the optimum pH and a limiting amount of SBP, as determined previously, were used. Each reactor contained 400 mg/L of PEG as in the previous set of experiments.

### **3B.3.4 PEG Dose**

The last parameter to be optimized was PEG dose. A 20 g/L stock solution of PEG was prepared and stored at room temperature. During this final set of experiments, the optimum pH and  $[\text{H}_2\text{O}_2]/[\text{Substrate}]$  were used, and the SBP dose in the reactors was kept at less than the optimum dose determined previously.

## **3B.4 Analytical Methods**

### **3B.4.1 Aromatic Compound Concentration Assay**

The concentrations of the aromatic compounds were determined by either the Direct spectrophotometric method or the colorimetric method. The concentrations of the substrates are expressed as molar quantities, where one millimolar is equal to 94 mg/L of phenol and 108 mg/L of cresols. The colorimetric method has been described earlier in Section 3A.4.1. It was observed



that the colorimetric method could be used for phenols and all of the cresols except p-cresol, in which case the direct spectrophotometric method was used.

The direct spectrophotometric method was based on the absorbance of ultraviolet (u.v.) light by phenols. Phenol derivatives absorb u.v. light with maxima between 270 and 284 nm in a quantity directly proportional to the concentration of phenol derivatives in the solution. It has been reported that peroxidase, hydrogen peroxide and the buffer solutions used to maintain pH which do not interfere in this method (Klibanov, 1980). However, it was observed that the reaction products, which probably were different after the addition of additives, might interfere with the accuracy of this method (Wu. Y. *et al.*, 1997). The phenomenon was observed for p-cresol. It was suggested that the addition of alkali (NaOH) to the sample after the reaction might prevent this interference during analysis by u.v. method. For p-cresol samples which remained turbid after centrifugation and syringe filtration, 0.2 M NaOH was added in equal proportion and the absorbance was measured at 296 nm, the maximum of p-cresol sodium salt.

#### **3B.4.2 Peroxidase Activity Assay (same as 3A.4.2)**

#### **3B.5 Sources of Error**

In any experiment, many errors occur which may affect the reliability of the results. Systematic errors are due to analytical techniques and instruments, whereas random (or human) errors are due to personal carelessness. Calibration curves were done several times and compared to verify accuracy. A set of experiments, the hydrogen peroxide dose series for p-cresol, was repeated 3 times and the results were compared to determine the reliability of the experimental results.

As previously mentioned, the products of the enzymatic reaction are mostly phenols. If a portion of these reaction products remained in solution, they would absorb at the same wavelength as the compound being analyzed, and could

therefore lead to errors in the estimation of the amount of initial substrate that actually remained in solution.

## **4. RESULTS AND DISCUSSION**

### **4A. Extraction and characterization of crude soybean peroxidase enzyme**

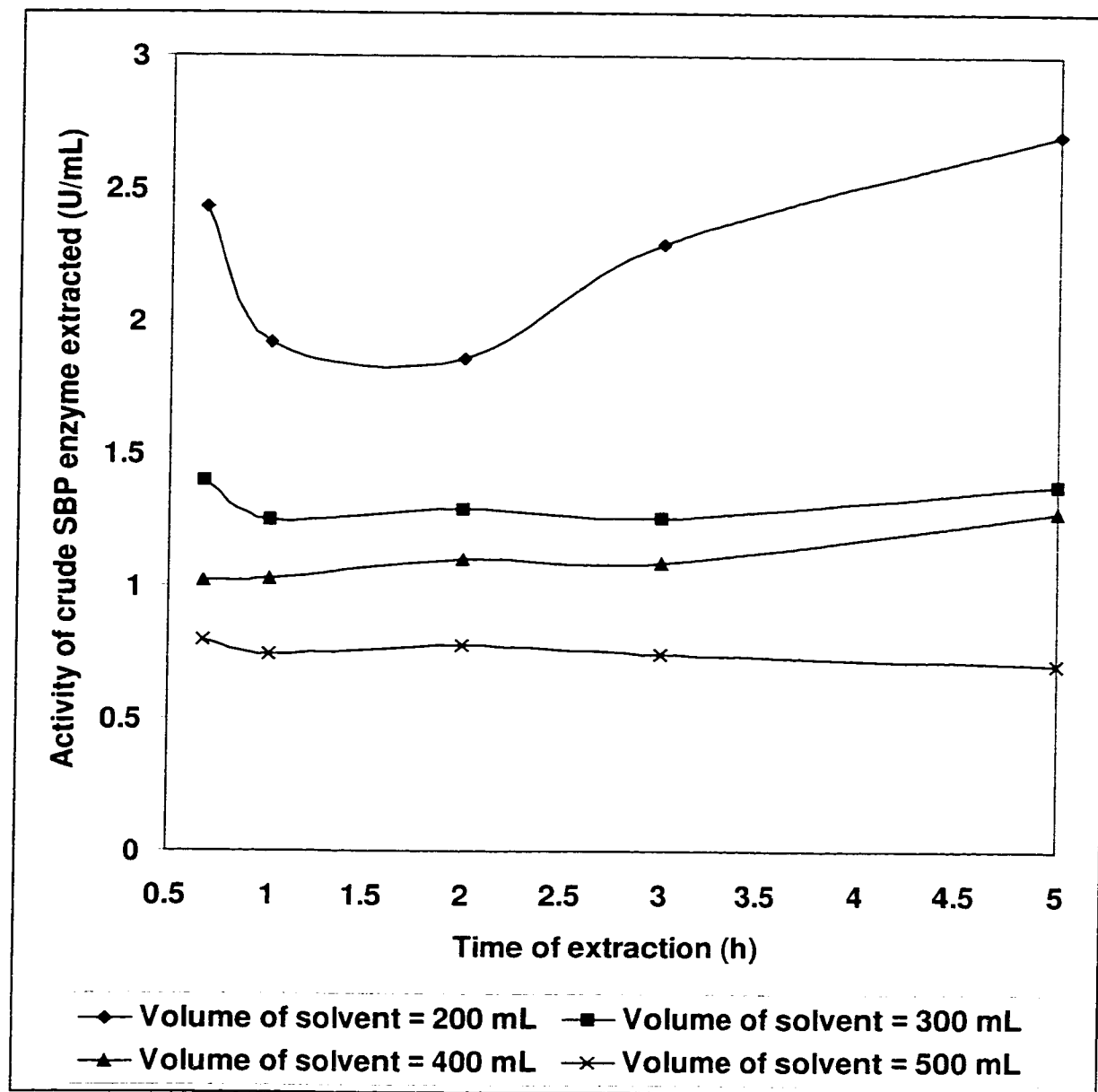
The experiments in this study were designed to develop a suitable experimental protocol for the extraction of soybean peroxidase (SBP) from soybean hulls and determine some characteristic properties of the extracted enzyme like its temperature stability, hydrogen peroxide demand and phenol content in the crude enzyme.

#### **4A.1 Extraction of soybean peroxidase from soybean hulls**

The parameters that were optimized were hulls to solvent ratio, choice of solvent and time of extraction of enzyme. Figures 4A.1.1 to 4A.1.3 show the results obtained.

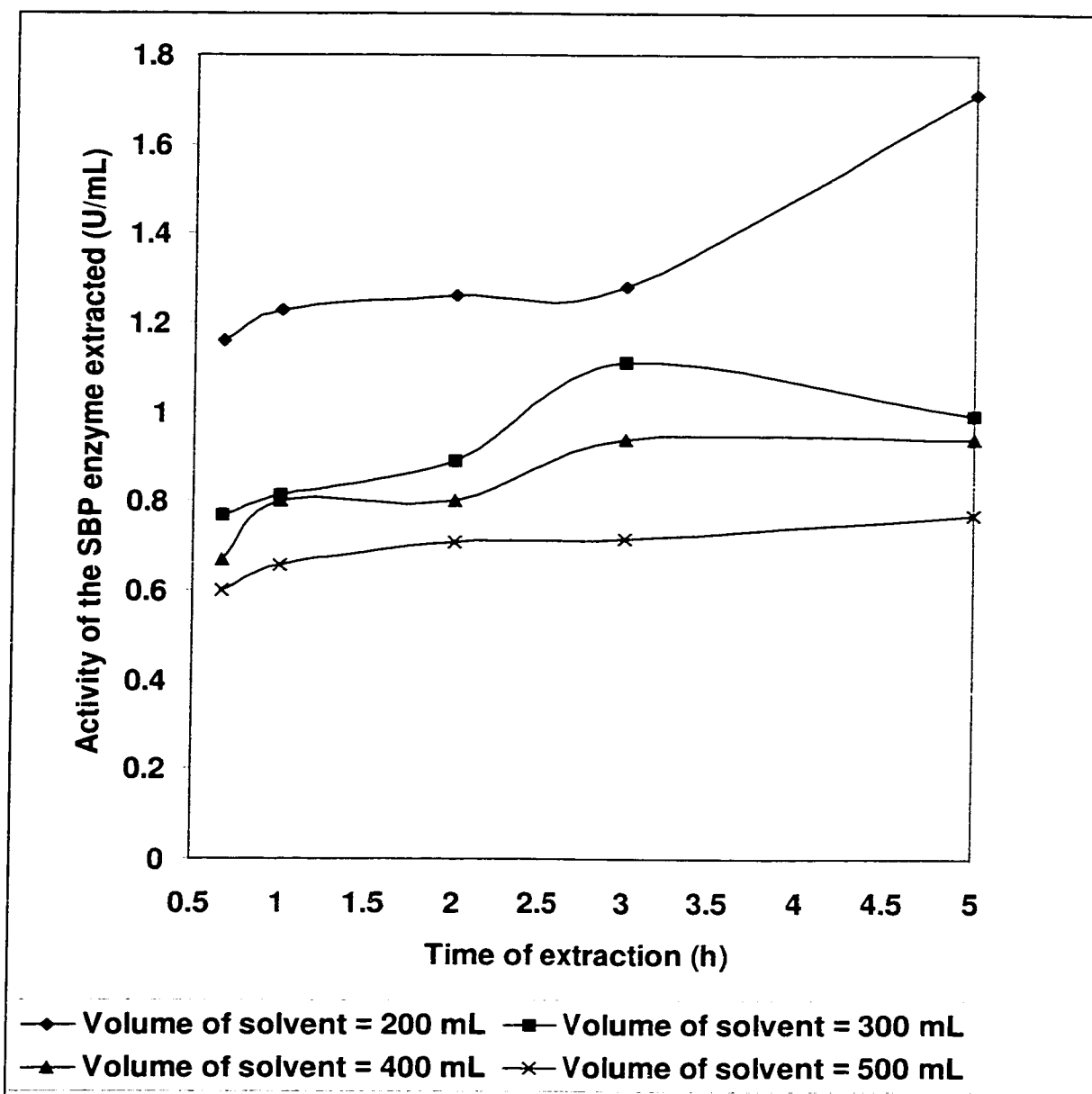
Extraction of enzyme was almost complete in less than 40 minutes for both tap water and phosphate buffer as solvent. On reducing the amount of solvent, the enzyme extracted was more concentrated with more activity. However, the total units of enzyme extracted (50 and 80 units per gram of soybean hulls for enzyme A and B respectively) was not dependent on the volume of the solvent as shown in Figures 4A.1.1 and 4A.1.2. This was noticed for both the crude enzymes A and B (Figure 4A.1.3). This may be due to the fact that with the volume of solvent used the extraction was complete. Thus there is no optimum hulls to solvent ratio for the crude SBP extraction. In order to obtain concentrated enzyme in solution, hulls to solvent ratio should be high. Thus, the hulls to solvent ratio can be selected.

It is also evident from Figure 4A.1.2 that less amount (total units of enzyme) of enzyme was obtained after extraction, by using buffer solution as solvent, as compared to tap water as solvent. This could be due to lower solubility of the enzyme in the buffer solution, which contained a significant amount of salt in itself and thus had considerable less capacity to extract enzyme as compared to tap water.



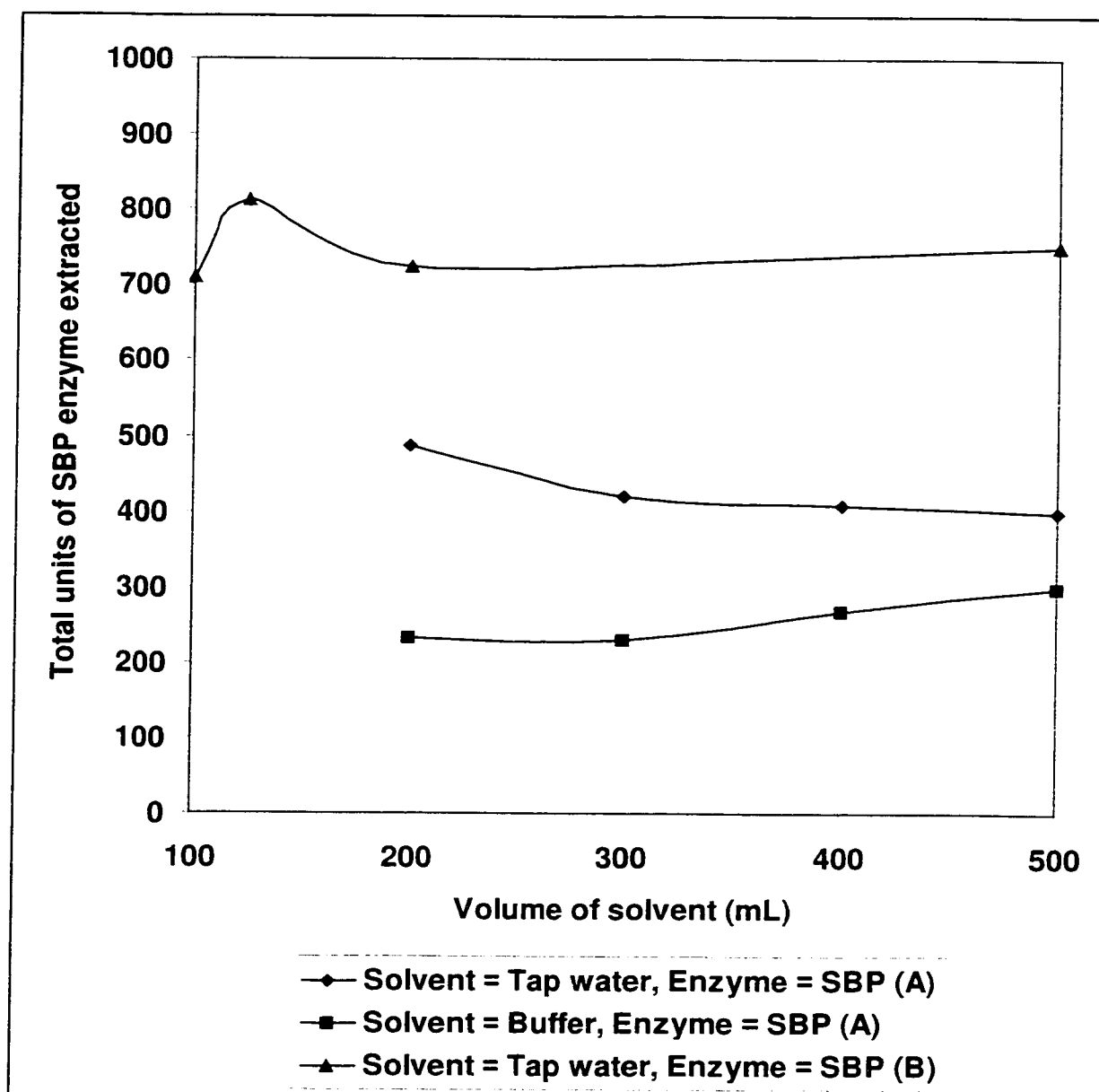
**CONDITIONS**  
 Soybean hulls used = 10 g  
 Solvent = Tap water  
 Room temperature = 22 °C

**Figure 4A.1.1 Effect of extraction time on the activity of crude SBP**  
 (A) extracted using tap water as solvent



**CONDITIONS**  
 Soybean hull used = 10 g  
 Solvent = Phosphate buffer  
 (0.1 M) (pH = 7.4)  
 Room temperature = 22 °C

**Figure 4A.1.2: Effect of extraction time on the activity of crude SBP  
 (A) extracted using Phosphate buffer as solvent**



**CONDITIONS**  
Time of mixing = 40 min  
Room temperature = 22 °C

**Figure 4A.1.3: Effect of the volume of solvent used on the total units of enzyme extracted**

Figure 4A.1.3 also shows that the source of enzyme hulls plays a critical role in the yield of the crude enzyme. SBP (B) whose hulls came from a different batch than the hulls of SBP (A) yields almost twice as much total enzyme activity units than that from the hulls of SBP (A). Thus the yield of the enzyme depends upon the source of the soybean hulls.

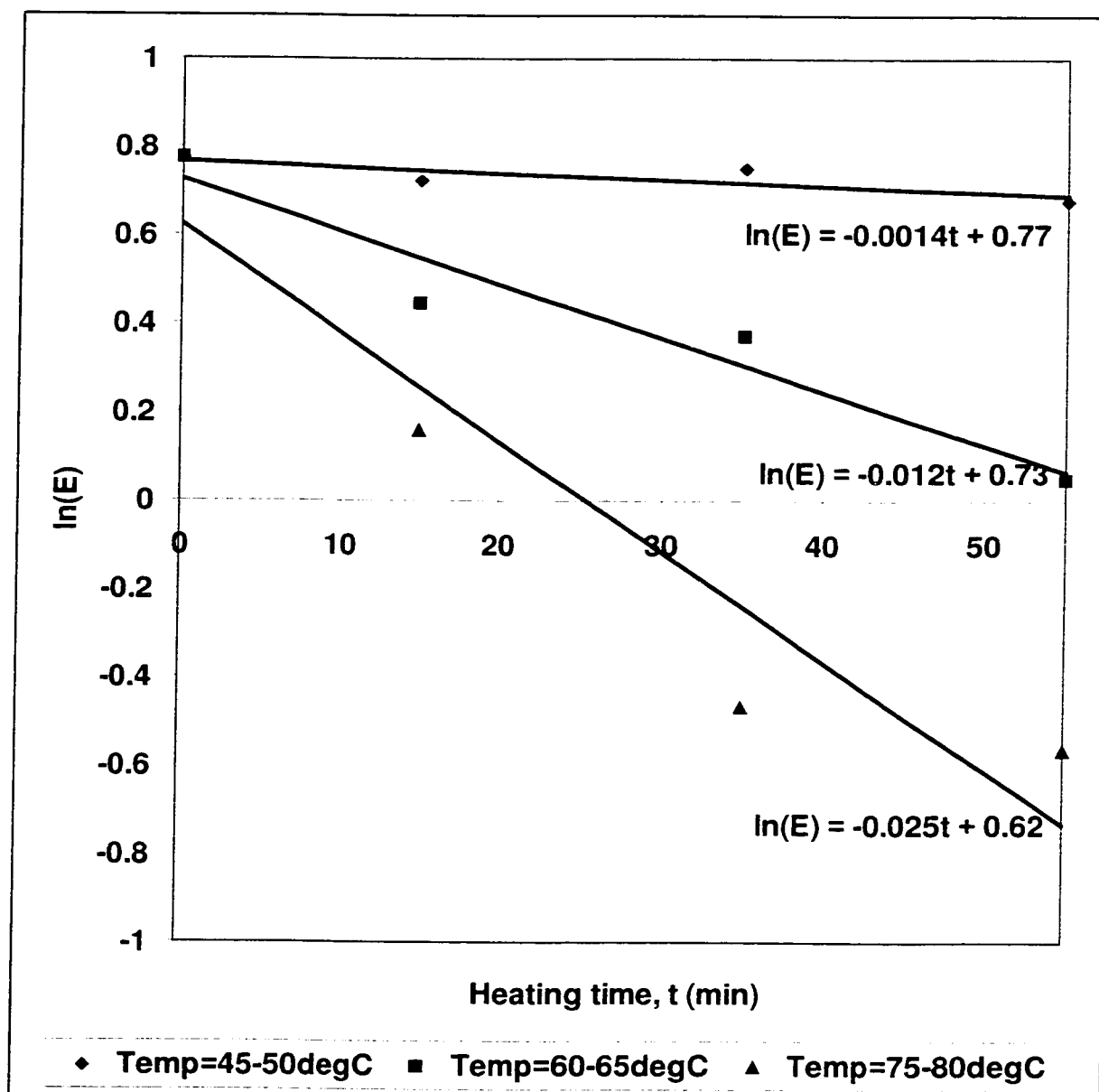
#### 4.A.2 Temperature stability of SBP

Figure 4A.2.1 shows that the activity of commercial SBP enzyme did not change at temperature range of 45-50°C. However above this temperature range it became unstable and the enzyme activity decreased considerably both with an increase in heating and an increase in heating temperature. The activity of the crude SBP (A) remained unchanged on heating up to a temperature range of 75-80°C for almost an hour. Thus it was highly stable as compared to the commercial SBP which started losing stability above 50°C.

The activity of crude SBP (B) also remained unchanged up to the temperature range 75-80°C. However at 90-95°C temperature range, it lost its activity rapidly with time. Therefore, the crude SBP (B) was found to be stable until a temperature of about 80°C. The half life of the enzyme at various temperature ranges was determined from Figures 4A.2.1 and 4A.2.2, where  $\ln(\text{enzyme activity})$  is plotted against the time of heating (min). The basic equations used for determining the half life are given in Section 1.1.3.

The slope of the curve gives rate constant “-k” and the **Half Life** is given by  $[\ln 2]/[k]$  (Section 1.1.3). The half life and rate constant values are given in Table 4A.2.

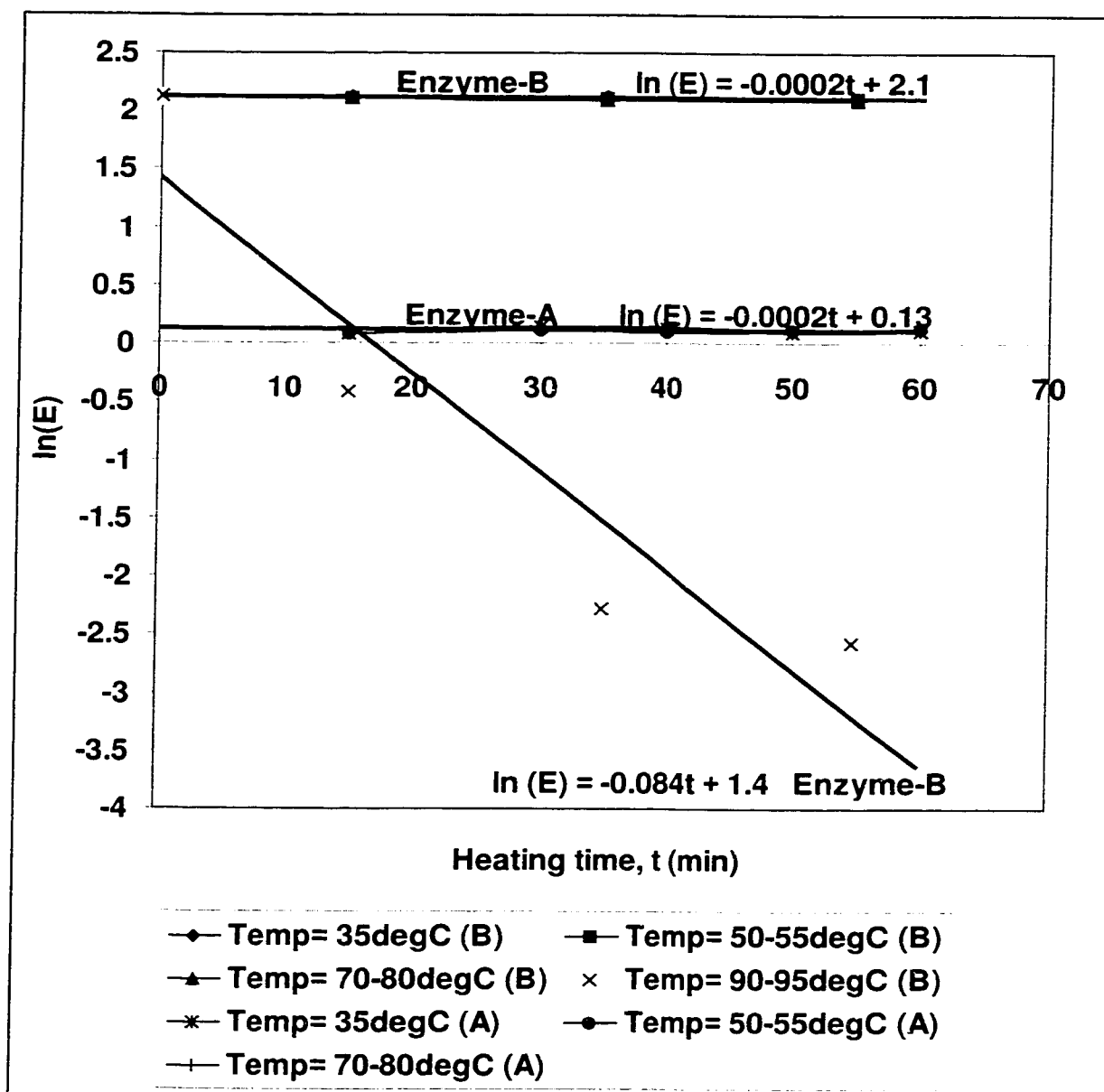
The rate constants of crude SBP enzyme A and B are almost zero below 80°C and hence its half lives are very high. At 90°C the activity of the crude SBP enzyme B decreased rapidly and its half life was calculated to be as low as 8.2 minutes. On comparing the half life, it is evident that the crude SBP enzyme A and B are more stable than commercial SBP enzyme at temperatures below 80°C.



$E$  = Activity of commercial SBP (U/mL)

**Figure 4A.2.1: Variation of the activity of the commercial SBP with heating time**





**E = Activity of crude SBP (A & B)**

**(A) and (B) in legend denotes Enzyme A and B respectively**

**Figure 4A.2.2: Variation of the activity of the crude SBP (A & B) with heating time**

**Table 4A.2 Half life of commercial and crude SBP (B) at different temperature ranges**

| Type of enzyme | Temperature range ( <sup>0</sup> C) | Rate Constant "k" (min <sup>-1</sup> ) | Half Life (min) |
|----------------|-------------------------------------|--|-----------------|
| Commercial SBP | 45—50                               | 0.0014                                 | 495             |
| Commercial SBP | 60—65                               | 0.012                                  | 58              |
| Commercial SBP | 75—80                               | 0.025                                  | 28              |
| Crude SBP (A)  | 35                                  | 0.0002                                 | 3465            |
| Crude SBP (A)  | 50—55                               | 0.0002                                 | 3465            |
| Crude SBP (A)  | 70—80                               | 0.0002                                 | 3465            |
| Crude SBP (B)  | 35                                  | 0.0002                                 | 3465            |
| Crude SBP (B)  | 50—55                               | 0.0002                                 | 3465            |
| Crude SBP (B)  | 70—80                               | 0.0002                                 | 3465            |
| Crude SBP (B)  | 90—95                               | 0.084                                  | 8.2             |

A modified Arrhenius Equation, called the Vant Hoff equation is often used to report the temperature effect on rate constant. It is given by

$$\frac{k_2}{k_1} = \theta^{T_2 - T_1}$$

where  $\theta$  = Constant

$T_2, T_1$  = Temperatures

$k_2$  = Rate constant at temperature  $T_2$

$k_1$  = Rate constant at temperature  $T_1$

For commercial SBP enzyme,

$k_1 = 0.012 \text{ min}^{-1}$  at  $T_1 = 62.5^\circ\text{C}$  and

$k_2 = 0.025 \text{ min}^{-1}$  at  $T_2 = 77.5^\circ\text{C}$

For crude SBP (B),

$k_1 = 0.0002 \text{ min}^{-1}$  at  $T_1 = 85^\circ\text{C}$  and

$k_2 = 0.084 \text{ min}^{-1}$  at  $T_2 = 92.5^\circ\text{C}$

By substituting the values in the above equation, one gets

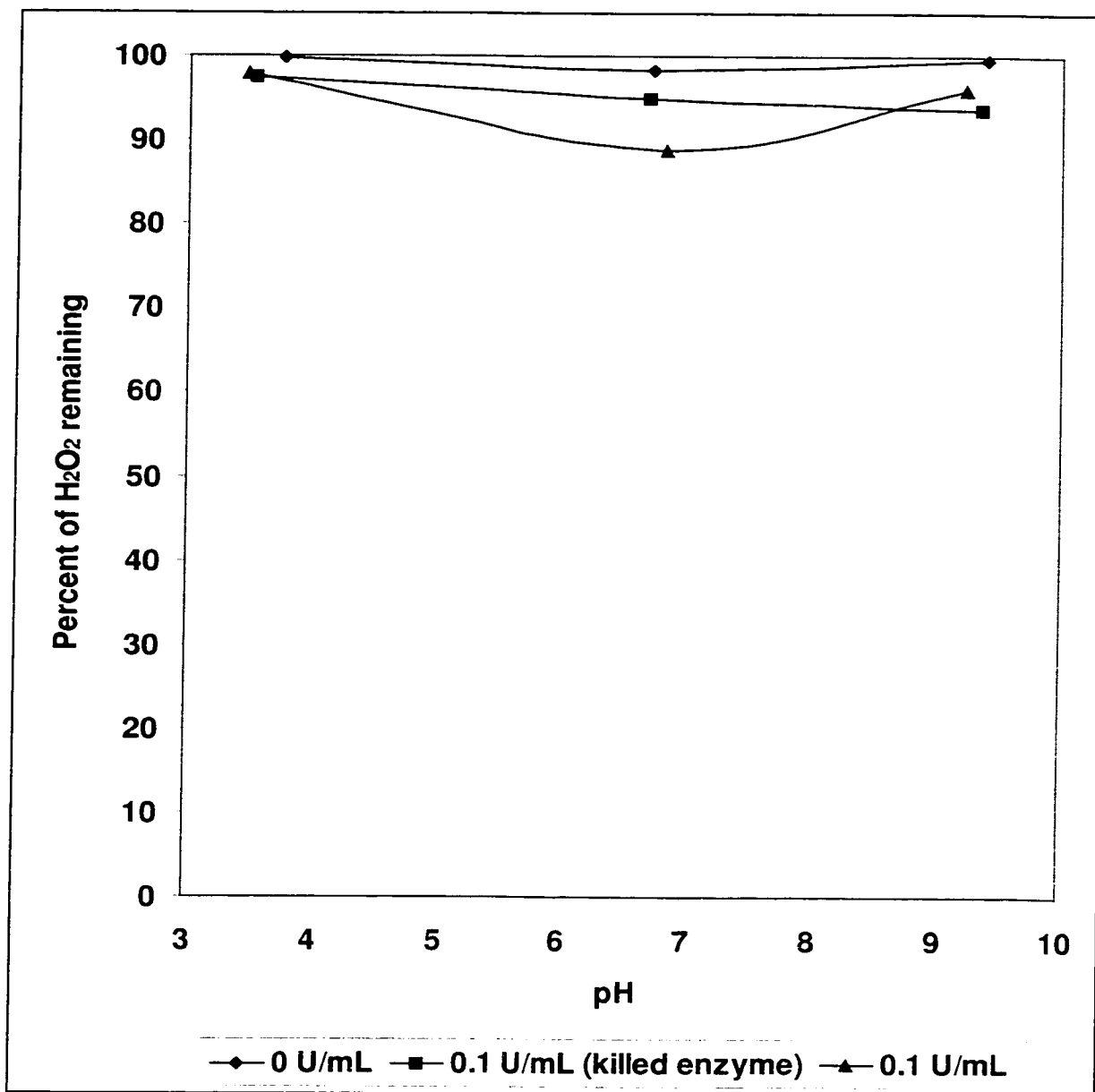
$\theta = 1.0493$  for commercial SBP and

$\theta = 2.238$  for crude SBP

$\theta$  is an important parameter in wastewater treatment often used for determining rate constants at different temperatures. In this context higher the value of  $\theta$ , higher is the rate of change in enzyme activity. The usefulness of  $\theta$  as an indicator for temperature influence is limited within a given range temperature. For commercial SBP the reported value of  $\theta$  is valid between temperatures of  $62.5^\circ\text{C}$  and  $77.5^\circ\text{C}$  and for crude SBP it is valid between  $85^\circ\text{C}$  and  $92.5^\circ\text{C}$ .

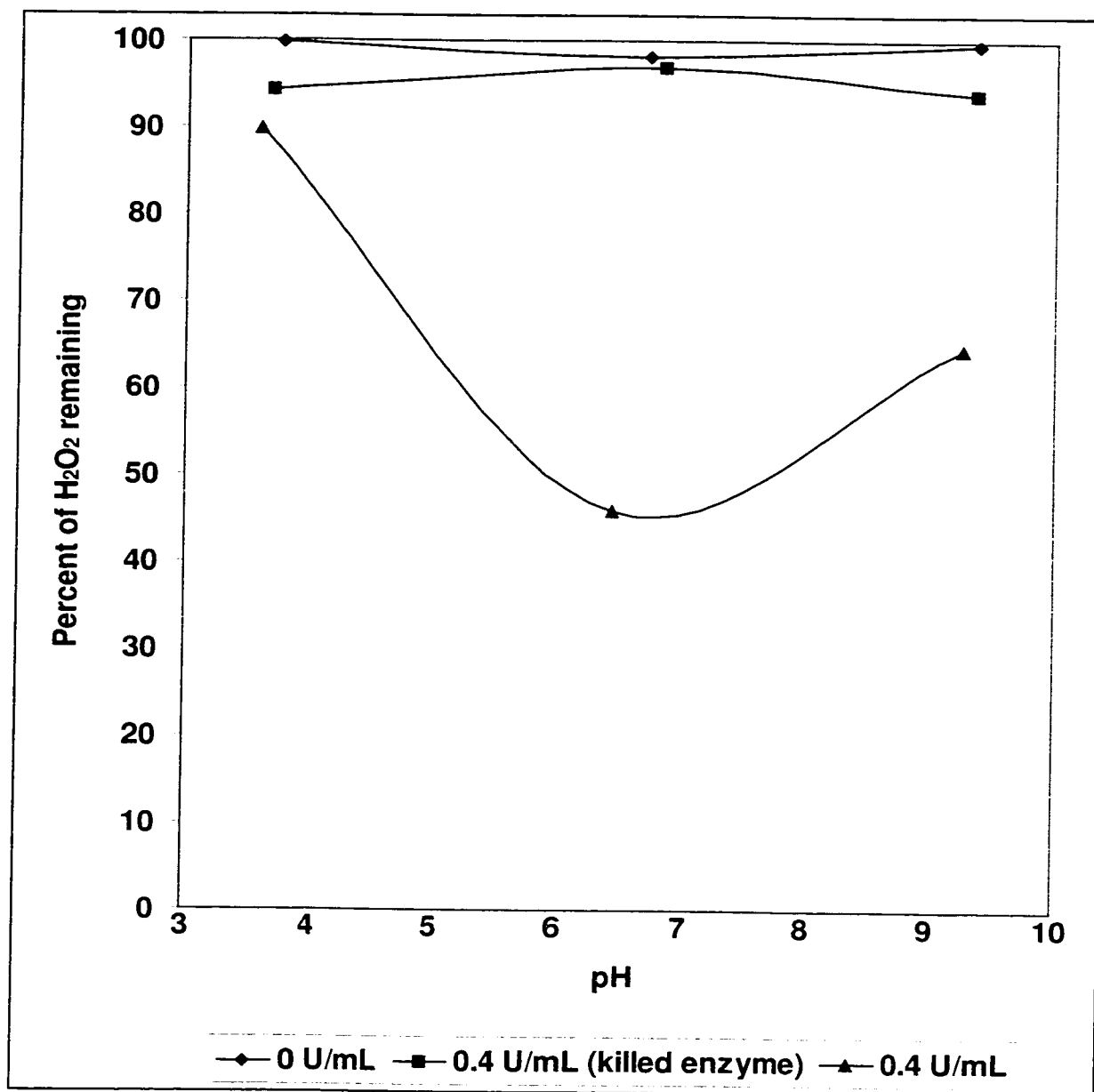
#### **4A.3 Hydrogen Peroxide demand of crude SBP**

The results obtained are summarized in Table 4A.3. At 0 U/mL of crude SBP (B), the hydrogen peroxide remaining after 3 h was almost 100 %. Similar results were obtained with thermally denatured crude SBP (B) at enzyme dosages of 0.1, 0.4 and 0.8 U/mL. However, for unheated crude SBP (B), the residual hydrogen peroxide decreased with an increase in enzyme dosage. Only 12 % of hydrogen peroxide remained when enzyme dose was 0.8 U/mL. Thus, the crude SBP (B) had significant hydrogen peroxide demand and it increased with an increase in the activity of the enzyme.



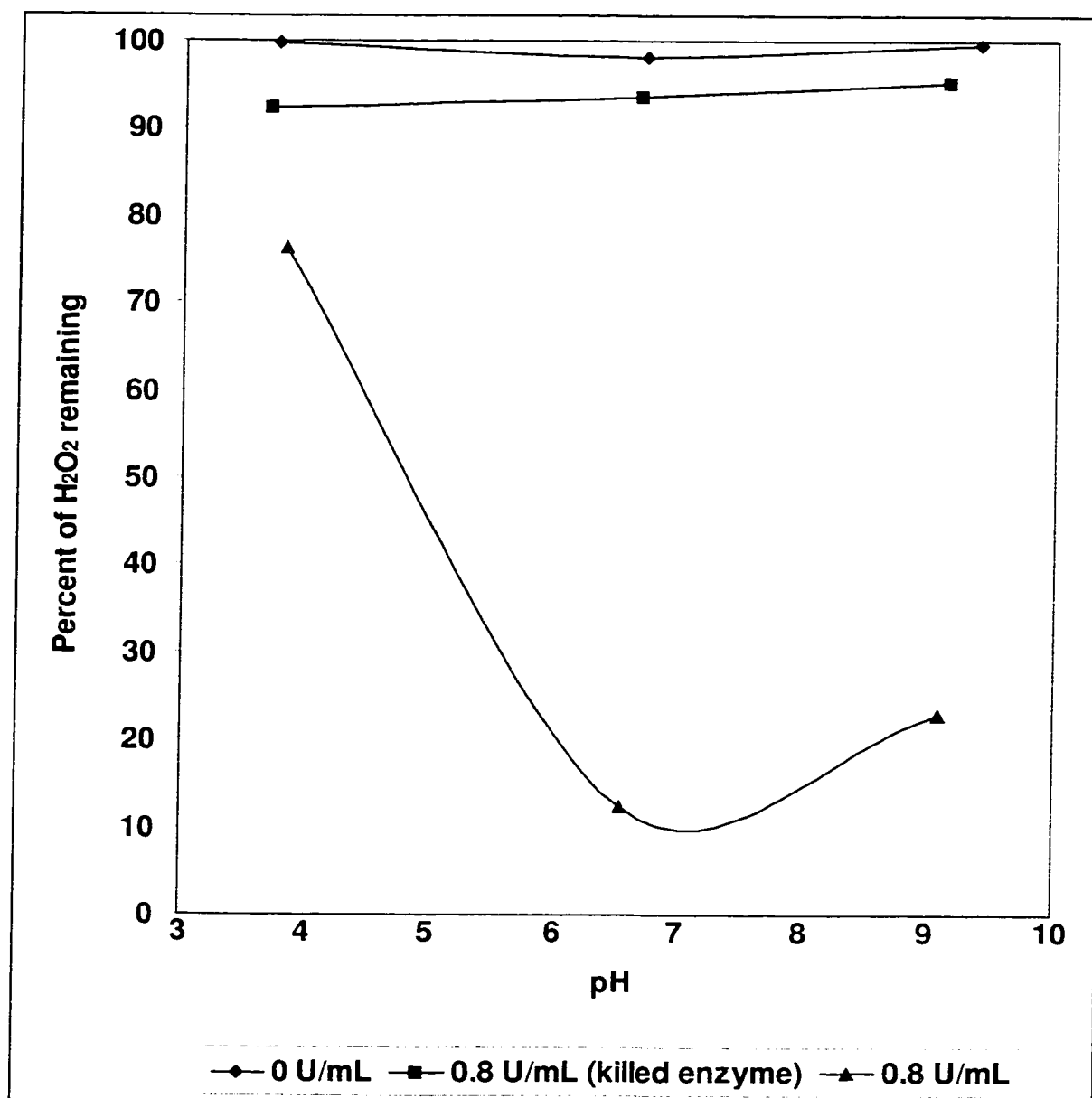
**CONDITIONS**  
 $\text{H}_2\text{O}_2$  dose = 1 mM  
SBP dose = 0.1 U/mL  
pH = 3---9  
Time of mixing = 3 hrs

**Figure 4A.3.1: Effect of pH on  $\text{H}_2\text{O}_2$  demand of crude SBP (B)  
at an enzyme dose of 0.1 U/mL**



**CONDITIONS**  
H<sub>2</sub>O<sub>2</sub> dose = 1 mM  
SBP dose = 0.4 U/mL  
pH = 3—10  
Time of mixing = 3 hrs

**Figure 4A.3.2: Effect of pH on H<sub>2</sub>O<sub>2</sub> demand of crude SBP (B)**  
**at an enzyme dose of 0.4 U/mL**



**CONDITIONS**  
H<sub>2</sub>O<sub>2</sub> dose = 1 mM  
SBP dose = 0.8 U/mL  
pH = 3—10  
Time of mixing = 3 hrs

**Figure 4A.3.3: Effect of pH on H<sub>2</sub>O<sub>2</sub> demand of crude SBP  
at an enzyme dose of 0.8 U/mL**

**Table 4A.3: Effect of SBP dose on the H<sub>2</sub>O<sub>2</sub> demand of the enzyme at pH 6-7**

| Sl No. | Crude SBP (B) | Enzyme activity (U/mL) | Percent of H <sub>2</sub> O <sub>2</sub> remaining |
|--------|---------------|------------------------|--|
| 1      | Control       | 0                      | 98   |
| 2      | Active        | 0.1                    | 89   |
| 3      | Active        | 0.4                    | 46   |
| 4      | Active        | 0.8                    | 12   |
| 5      | Denatured     | 0 (originally 0.1)     | 95   |
| 6      | Denatured     | 0 (originally 0.4)     | 97   |
| 7      | Denatured     | 0 (originally 0.8)     | 94   |

Figures 4A.3.1 to 4A.3.3 show the effect of pH on H<sub>2</sub>O<sub>2</sub> demand. It is observed that the hydrogen peroxide demand is maximum in the pH range of 6—7. Thus, pH plays a critical role in exerting a hydrogen peroxide demand by the enzyme.

The H<sub>2</sub>O<sub>2</sub> demand of the 30 mL mixture in the batch containers can be due to three possible reasons. Firstly, it may be due to the organic matter present in the mixture that is susceptible to oxidation by the hydrogen peroxide added to it. Due to the oxidation of the organic matter the H<sub>2</sub>O<sub>2</sub> decomposes and thus the latter is lost from the mixture accounting for the significant H<sub>2</sub>O<sub>2</sub> demand. Secondly, it may be due to the phenol content in the crude SBP. The crude enzyme may contain a certain amount of phenolics which may react with the H<sub>2</sub>O<sub>2</sub> in presence of the enzyme to form other reaction products. So the H<sub>2</sub>O<sub>2</sub> may be consumed because of this reaction. Finally, the H<sub>2</sub>O<sub>2</sub> demand may be due to

the catalase present in the crude SBP. Catalases are found in all plants. They are, both structurally and functionally, close relations of the peroxidases. They are easily crystallizable haem enzymes with four iron atoms per molecule attached to protein. Catalases decompose hydrogen peroxide to oxygen and water.

From, the results obtained we find that the thermally denatured enzyme had hardly any  $\text{H}_2\text{O}_2$  demand after 3 hrs. So, the organic matter present in the mixture was not the reason for the significant  $\text{H}_2\text{O}_2$  demand. Also, we found (Section 4A.4 below) that the phenol present in the crude SBP was so small, that it was impossible for the  $\text{H}_2\text{O}_2$  demand of the mixture to be due to it. Therefore, we are left with the only possibility, catalase present in the crude SBP. Thus, the crude SBP (B) had significant  $\text{H}_2\text{O}_2$  demand and it was due to the consumption of  $\text{H}_2\text{O}_2$  by the catalase present in the enzyme.

#### **4A.4 Phenol content in crude SBP**

Phenol content in crude SBP (A) is found to be  $62.4 \mu\text{M}$  and that in crude SBP enzyme B was found to be  $164 \mu\text{M}$ . Consequently, when the crude SBP enzymes were used for the removal of phenolic compounds from wastewater using hydrogen peroxide it introduced a certain amount of phenol in the reaction. If the additional phenol due to the enzyme is significantly high, adjustments must be made in enzyme and hydrogen peroxide doses.

Table 4A.4 shows that with enzyme dosages as high as  $0.4 \text{ U/mL}$ , the phenolic compounds introduced in a  $30 \text{ mL}$  reactor was only  $18 \mu\text{M}$ . This is a small number when compared to the substrate concentration in the range of  $1000 \mu\text{M}$ . Therefore, the error introduced by the enzyme is less than  $1.8 \%$  for an enzyme dose of  $0.4 \text{ U/mL}$  and no correction for the phenolic content in the crude enzyme is necessary unless a high dose of enzyme is used for wastewater treatment.



**Table 4A.4: Phenol contributed by crude SBP**

| No. | SBP dosage<br>(U/mL) | Phenol contributed by<br>SBP (A)<br>( $\mu$ M) | Phenol contributed by<br>SBP (B)<br>( $\mu$ M) |
|-----|----------------------|--|--|
| 1   | 0.05                 | 1.0  | 2.3  |
| 2   | 0.1                  | 2.0  | 4.5  |
| 3   | 0.3                  | 6.1  | 14   |
| 4   | 0.4                  | 8.1  | 18   |
| 5   | 0.5                  | 10   | 23   |
| 6   | 0.8                  | 16   | 36   |

#### 4A.5 Error Analysis

A set of experiments was conducted in order to determine the reliability of the results in this study. The  $\text{H}_2\text{O}_2$  demand for the crude SBP doses of 0.1, 0.4 and 0.8 U/mL was determined in triplicate and the percent deviation was calculated. Each solution was prepared separately to determine the accuracy of analytical techniques. The test parameters were as follows: pH = 6—7,  $\text{H}_2\text{O}_2$  = 1 mM and crude SBP dose = 0.1, 0.4 and 0.8 U/mL. The results of these experiments are listed in Table 4A.5. These results indicate that the deviation was always less than  $\pm 4$  %, which is considered to be within an acceptable range. Therefore, the results obtained in this study are considered to be accurate and reliable.

**Table 4A.5: Error Analysis**

| <b>Sample #</b> | <b>Crude enzyme dose<br/>(U/mL)</b> | <b>Percent<br/>H<sub>2</sub>O<sub>2</sub> Remaining</b> | <b>Average</b>     |
|-----------------|-------------------------------------|---|--------------------|
| <b>1</b>        | <b>0.1</b>                          | <b>89.9</b>   | <b>91.0 ± 1.1</b>  |
| <b>2</b>        | <b>0.1</b>                          | <b>91.2</b>   |                    |
| <b>3</b>        | <b>0.1</b>                          | <b>92.0</b>   |                    |
| <b>4</b>        | <b>0.4</b>                          | <b>44.8</b>   | <b>44.8 ± 0.45</b> |
| <b>5</b>        | <b>0.4</b>                          | <b>45.3</b>   |                    |
| <b>6</b>        | <b>0.4</b>                          | <b>44.4</b>   |                    |
| <b>7</b>        | <b>0.8</b>                          | <b>11.6</b>   | <b>12 ± 0.44</b>   |
| <b>8</b>        | <b>0.8</b>                          | <b>12.1</b>   |                    |
| <b>9</b>        | <b>0.8</b>                          | <b>12.2</b>   |                    |

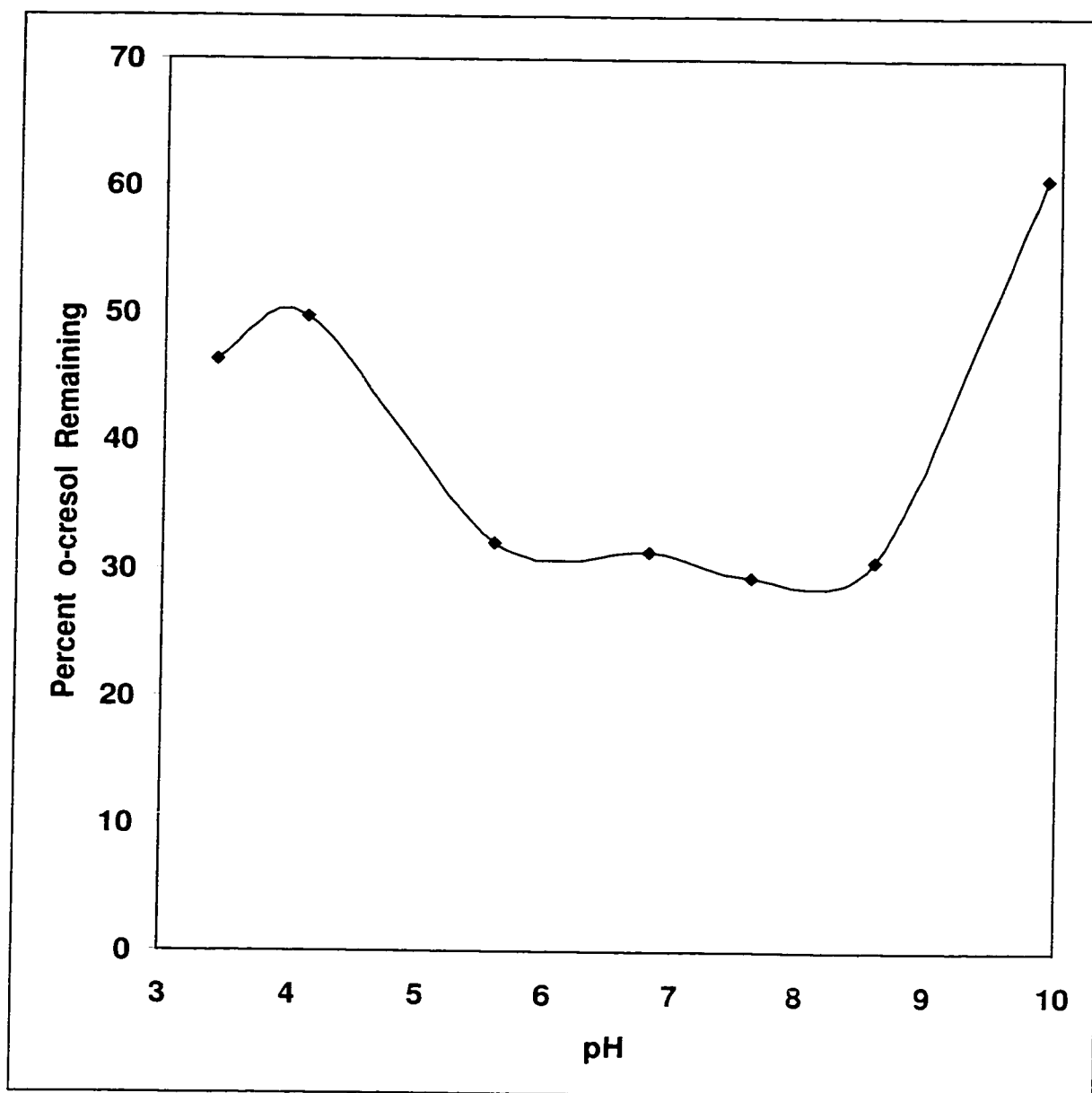
## 4B. Polymerization and removal of substrates

The experiments in this part were designed to achieve a removal of at least 95 % of the initial aromatic substrate concentration. The reaction parameters which were optimized were pH, soybean peroxidase (SBP) enzyme dose both in the presence and absence of polyethylene glycol (PEG), hydrogen peroxide to substrate ratio, ( $[H_2O_2]/[Substrate]$ ) and PEG dose. The effect of co-precipitation on crude SBP requirements was also investigated. Reactions were carried out once and mostly under stringent conditions so that the effect of the parameter being optimized is more pronounced. Earlier similar experiments were conducted by Wu. Y. *et al.* (1997) using horseradish peroxidase and Caza *et al.* (1999) by using commercially purified SBP enzyme. Both conducted their experiments at room temperature. However Wu Y *et al.* (1997) conducted their studies in buffered solutions and Caza *et al.* (1999) conducted their studies in unbuffered tap water.

It was observed that the reaction products which probably were different after the addition of additives, did not interfere with the accuracy of the method except for p-cresol. For p-cresol, the interference may be due to the formation of the Pummerer's ketone, a two ring compound proposed to result by reaction between p-cresol radical and a second molecule of p-cresol (Section 2.5). So, for p-cresol a modified method was suggested (Section 3B.4.1) which worked out well and did not interfere with the accuracy of the method.

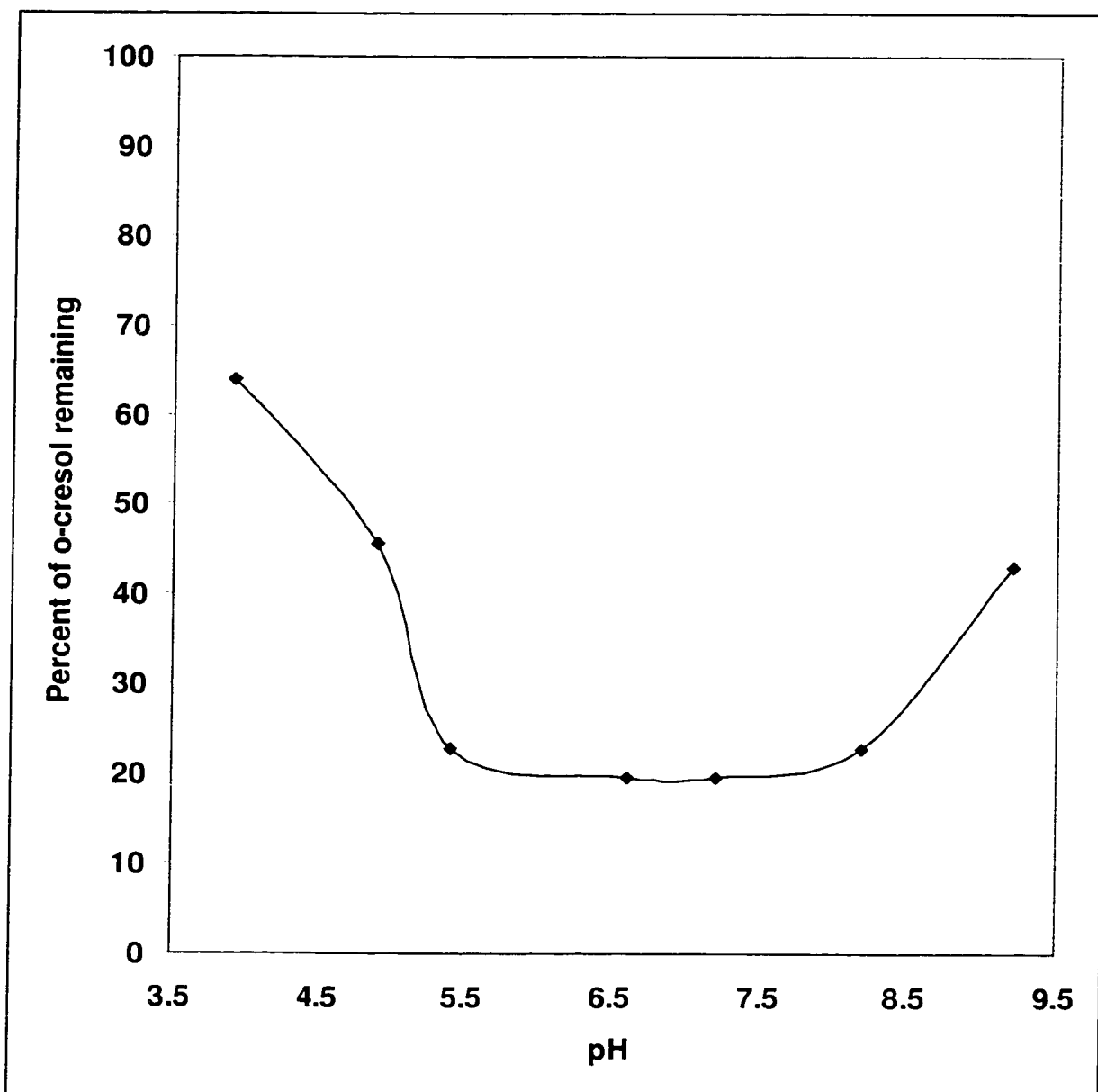
### 4B.1 pH

The optimum pH was determined for each substrate in the range of 3 to 10. The initial substrate concentration was 1.0 mM for all the compounds. The  $H_2O_2$  to substrate ratio was between 1.0 and 1.6 for each experiment and PEG was present in excess at 400 mg/L so that the removal efficiency was only dependent on pH and the enzyme dose. The reactions were stopped after 3 h which was



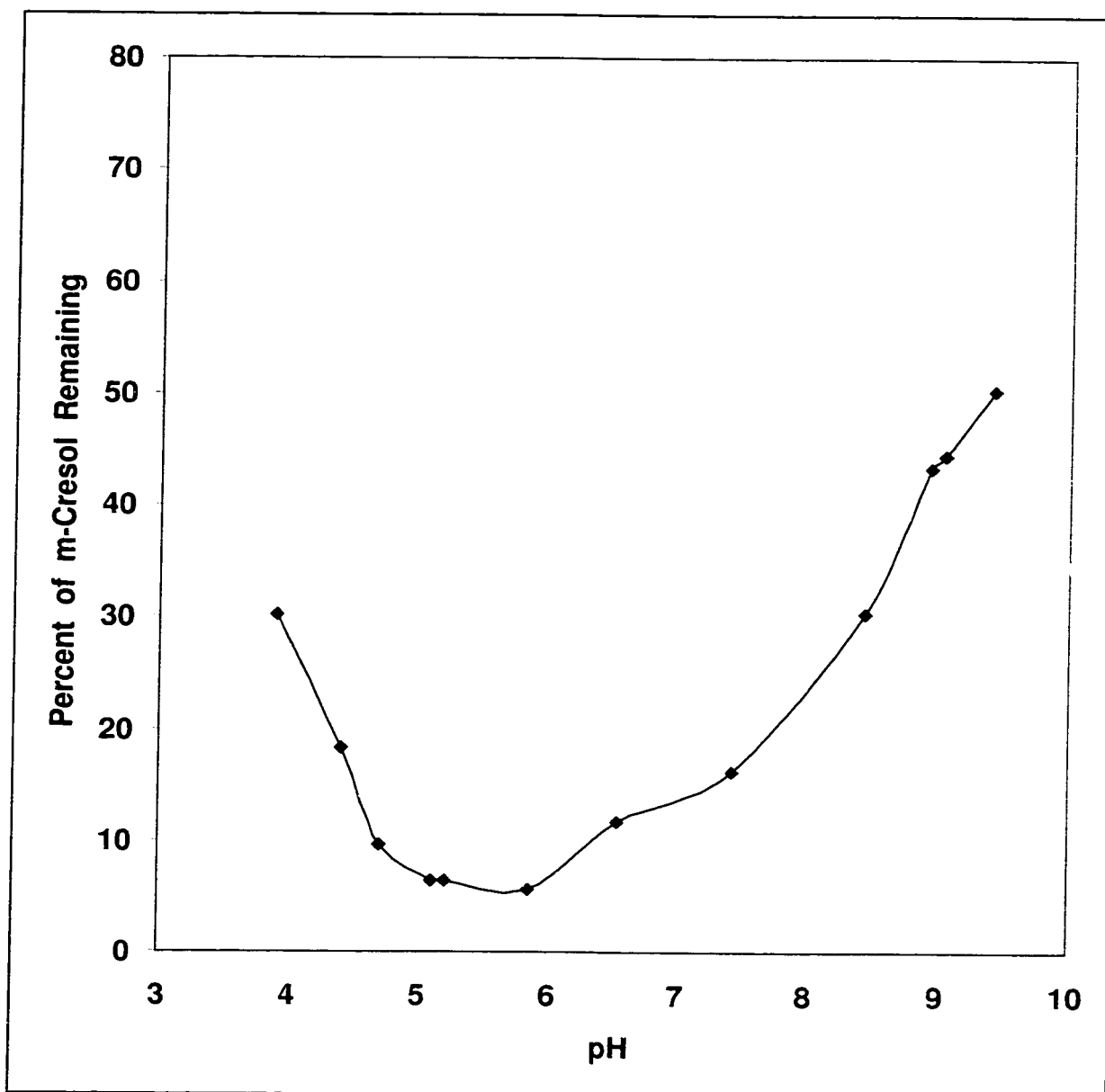
**Reactor Conditions**  
Substrate = 1mM  
[H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1mM/mM  
PEG = 400 mg/L  
SBP dose = 0.1 U/mL  
Reaction time = 3 h

**Figure 4B.1.1: Effect of pH on the removal of o-cresol  
using crude SBP (A)**



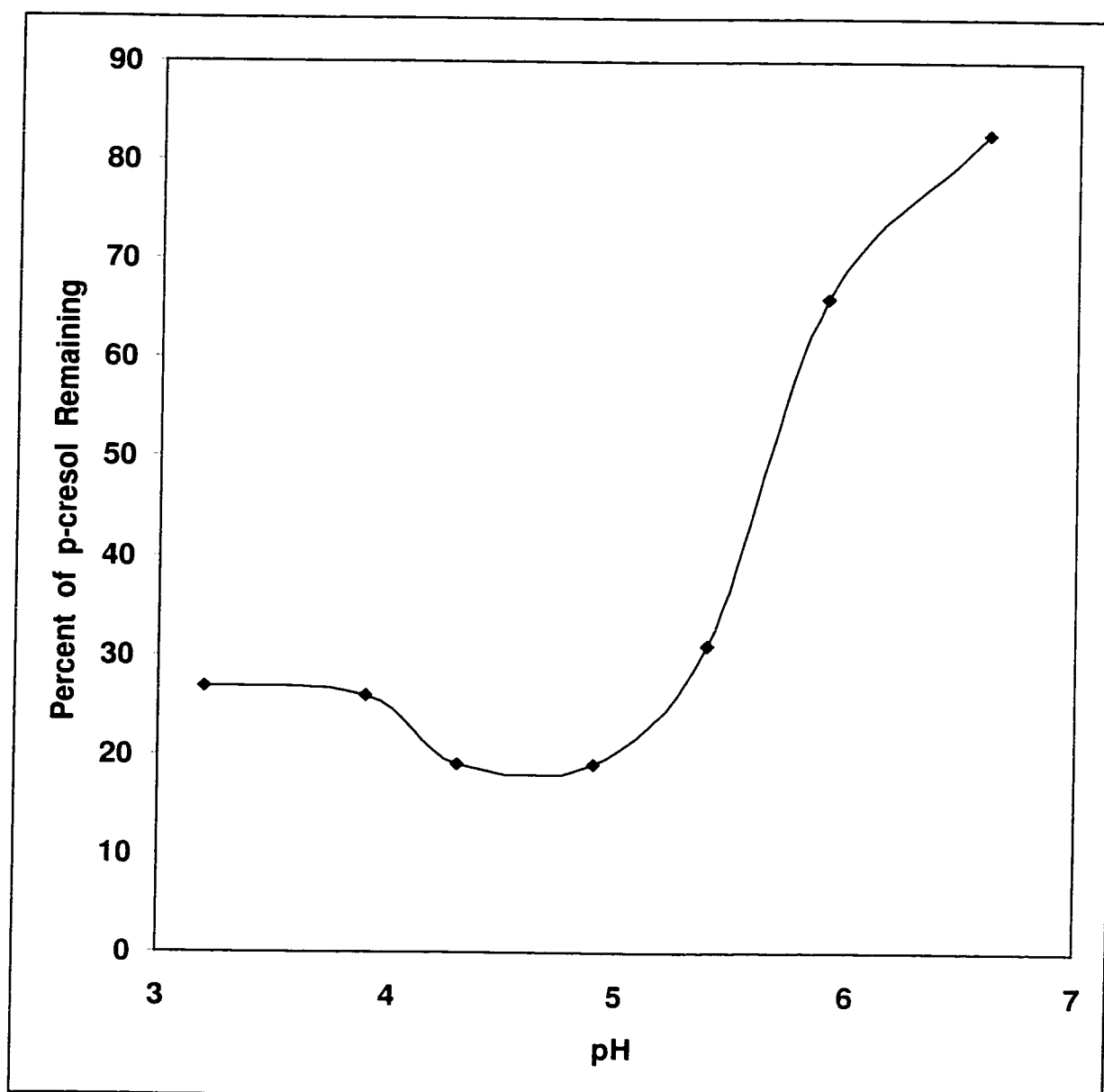
**Reactor Conditions**  
Substrate = 1 mM  
[H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1mM/mM  
PEG = 400 mg/L  
SBP dose = 0.15 U/mL  
Reaction Time = 3 h

**Figure 4B.1.2: Effect of pH on the removal of o-cresol using crude SBP (B)**



**Reactor Conditions**  
Substrate = 1 mM  
 $[\text{H}_2\text{O}_2]/[\text{Substrate}] = 1.2 \text{ mM/mM}$   
PEG = 400 mg/L  
SBP dose = 0.05 U/mL  
Reaction Time = 3 h

**Figure 4B.1.3: Effect of pH on the removal of m-Cresol  
using crude SBP (B)**



**Reactor Conditions**  
Substrate = 1 mM  
[H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1.6 mM/mM  
PEG = 400 mg/L  
SBP dose = 0.4 U/mL  
Reaction Time = 3 h

**Figure 4B.1.4: Effect of pH on the removal of p-cresol using crude SBP (B)**

considered to be sufficient time based on previous studies (Caza *et al.*, 1999). Removal efficiencies as a function of pH are presented in Figures 4B.1.1 through Fig 4B.1.4. These Figures show that o-cresol has a broad optimum pH range whereas both p-cresol and m-cresol have a relatively narrow optimum pH range. The pH is similar with both SBP enzymes A and B for reaction with o-cresol. P-cresol had an optimum pH range at 4—5 which is inconsistent from those found by Wu. Y. *et al* (1997) by using horseradish peroxidase and Caza *et al* (1999) by using commercially purified SBP enzyme. The optimum pH for each aromatic compound is listed in Table 4B.1.

**Table 4B.1: Optimum pH for cresols**

| <b>Substrate</b> | <b>Type of enzyme</b> | <b>Optimum pH range</b> | <b>Optimum pH</b> |
|------------------|-----------------------|-------------------------|-------------------|
| <b>o-cresol</b>  | <b>Crude SBP (A)</b>  | <b>5—8</b>              | <b>6.5</b>        |
| <b>o-cresol</b>  | <b>Crude SBP (B)</b>  | <b>5.5—8.5</b>          | <b>6</b>          |
| <b>m-cresol</b>  | <b>Crude SBP (B)</b>  | <b>5—6</b>              | <b>5.5</b>        |
| <b>p-cresol</b>  | <b>Crude SBP (B)</b>  | <b>4—5</b>              | <b>4.5</b>        |

Since reactions were carried out in unbuffered solutions, the pH after mixing was checked in order to verify that the reactions were carried out at the appropriate pH values. Typical results for o-cresol when using crude SBP enzyme B are shown in Table 4B.2. As expected, these results indicate that there was little change in pH values after the reaction was completed. The largest change in pH was 1.2 and the smallest was 0.1. The results obtained with other compounds were consistent with those obtained for this compound.



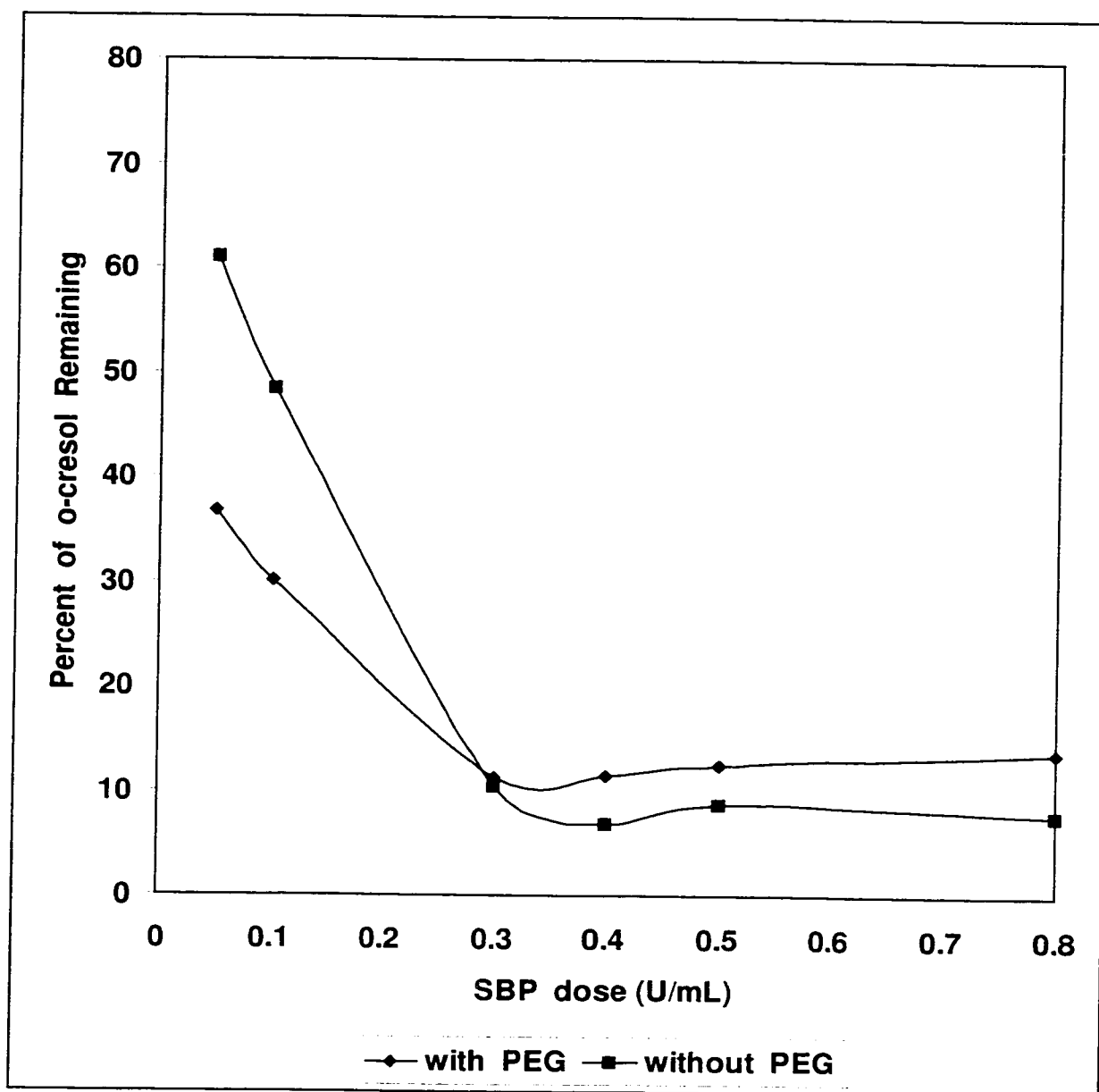
**Table 4B.2: Change in pH for o-cresol**

| Initial pH | Final pH |
|------------|----------|
| 3.9        | 5.1      |
| 4.9        | 5.4      |
| 5.4        | 5.9      |
| 6.6        | 6.7      |
| 7.2        | 7.1      |
| 8.2        | 8.4      |
| 9.2        | 9.1      |

#### **4B.2 Soybean Peroxidase Dose**

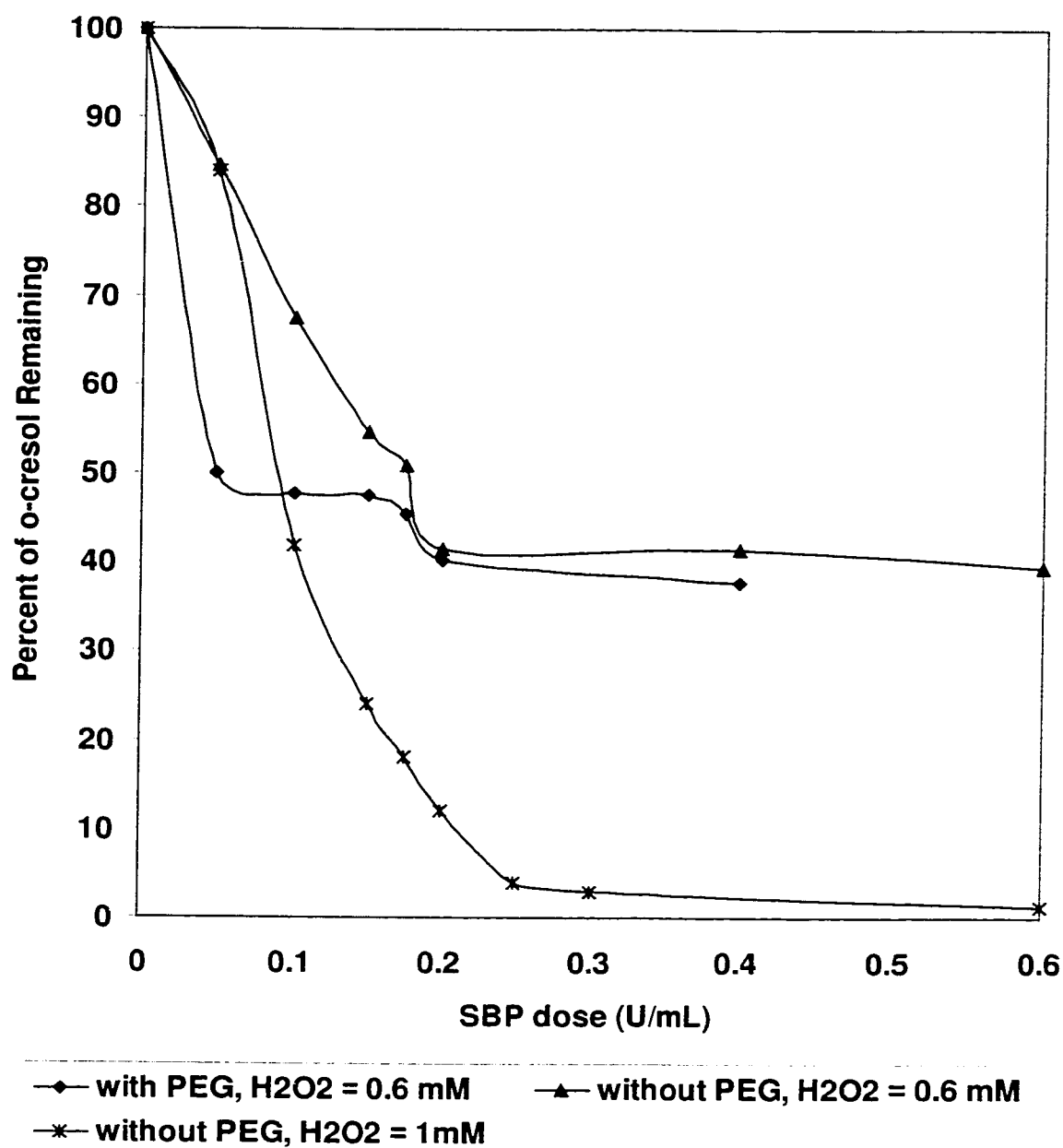
The experiments for optimum SBP dose series were conducted at the previously established optimum pH values shown in Table 4B.1. The optimum enzyme dose was determined both in the presence (400 mg/L) and in the absence of PEG. The results are presented in Figures 4B.2.1 to 4B.2.6. The  $[H_2O_2]/[Substrate]$  was kept between 0.6 and 2.2 mM/mM for different compounds.

Figure 4B.2.4 shows that 95 % removal in p-cresol could not be achieved even at a high enzyme dose of 0.6 U/mL. It was decided to increase the  $H_2O_2$  dose since it was suspected that due to the high  $H_2O_2$  demand of SBP the reaction may have run out of the required  $H_2O_2$ . However, it is observed from Figure 4B.2.4 that even after increasing the  $H_2O_2$  dose to 1.6 mM there was no improvement in the removal of the substrate. Similar experiments were conducted without PEG



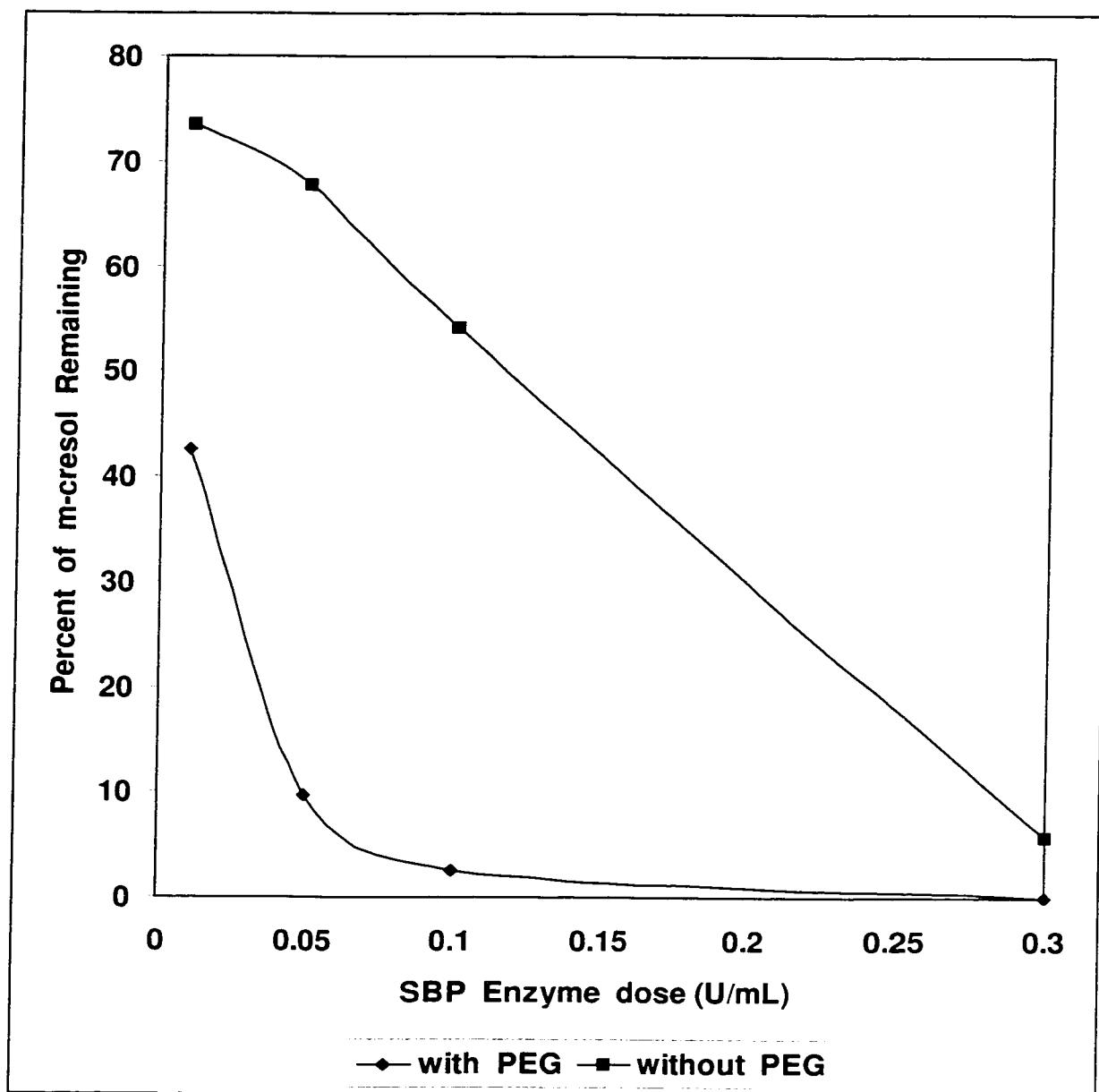
**Reactor Conditions**  
Substrate = 1 mM  
[H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1.2 mM/mM  
PEG = 400 mg/L  
SBP dose = 0.05–0.8 U/mL  
pH = 6–7  
Reaction Time = 3 h

**Figure 4B.2.1: Effect of crude SBP (A) dose on the removal of o-cresol**



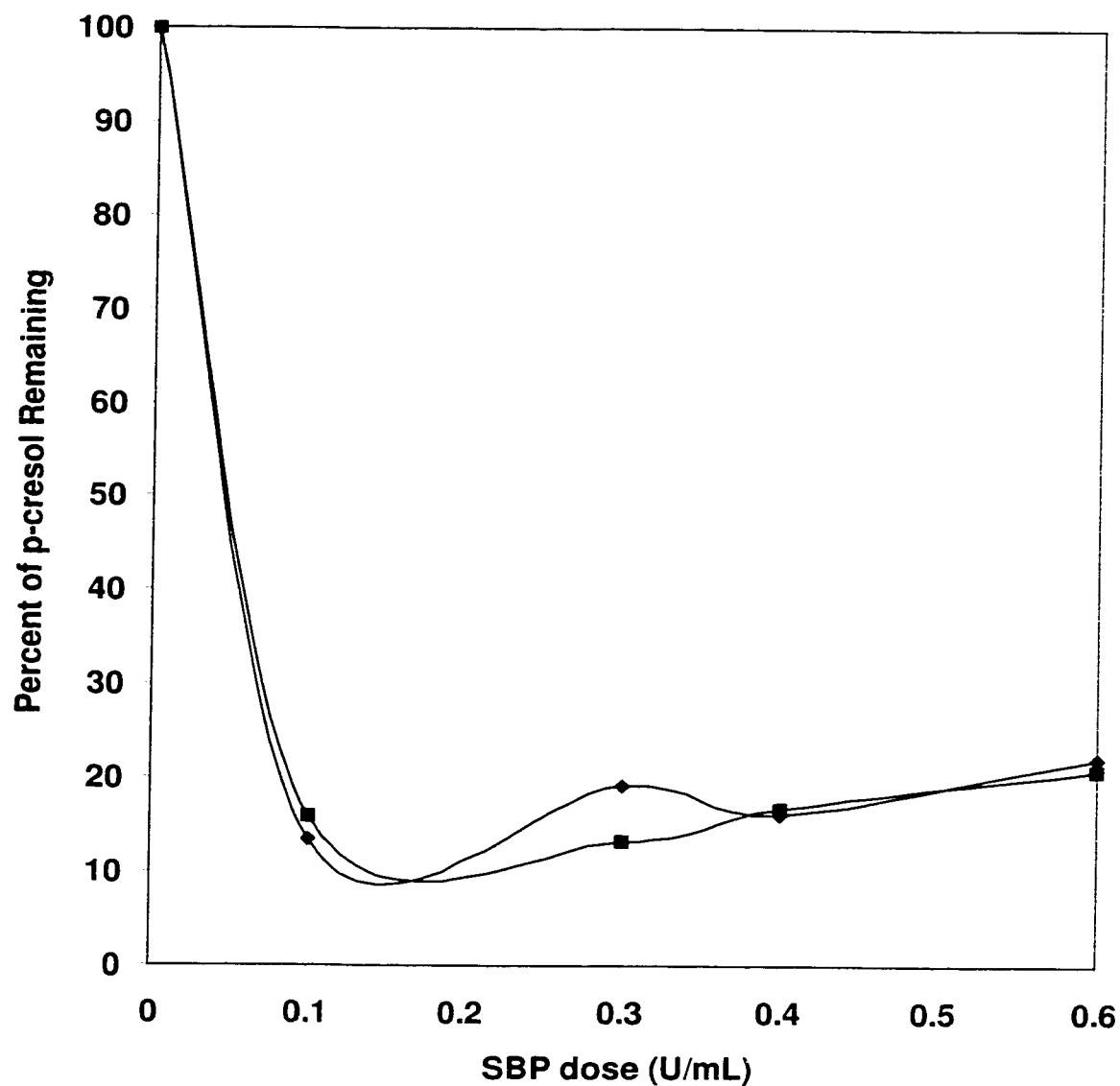
**Reactor Conditions**  
Substrate = 1 mM  
PEG = 400 mg/L  
H<sub>2</sub>O<sub>2</sub> = 0.6–1.0 mM  
pH = 6–7

**Figure 4B.2.2: Effect of crude SBP (B) dose on the removal of o-cresol**



**Reactor Conditions**  
Substrate = 1 mM  
pH = 5–5.5  
PEG = 400 mg/L  
[H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1.2 mM/mM

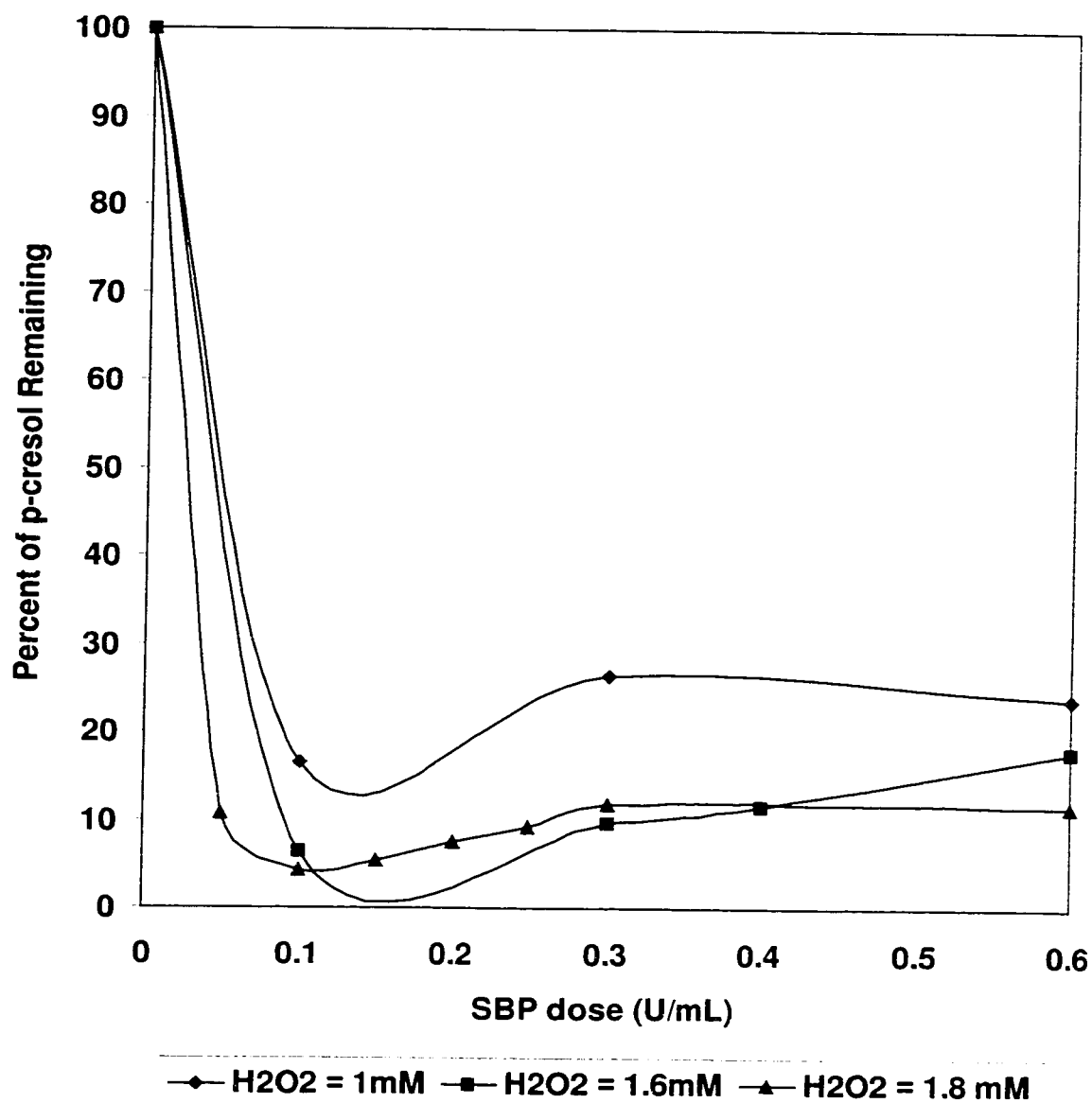
**Figure 4B.2.3: Effect of crude SBP (B) dose on the removal  
m-cresol**



—◆— H<sub>2</sub>O<sub>2</sub> = 1mM —■— H<sub>2</sub>O<sub>2</sub> = 1.6 mM

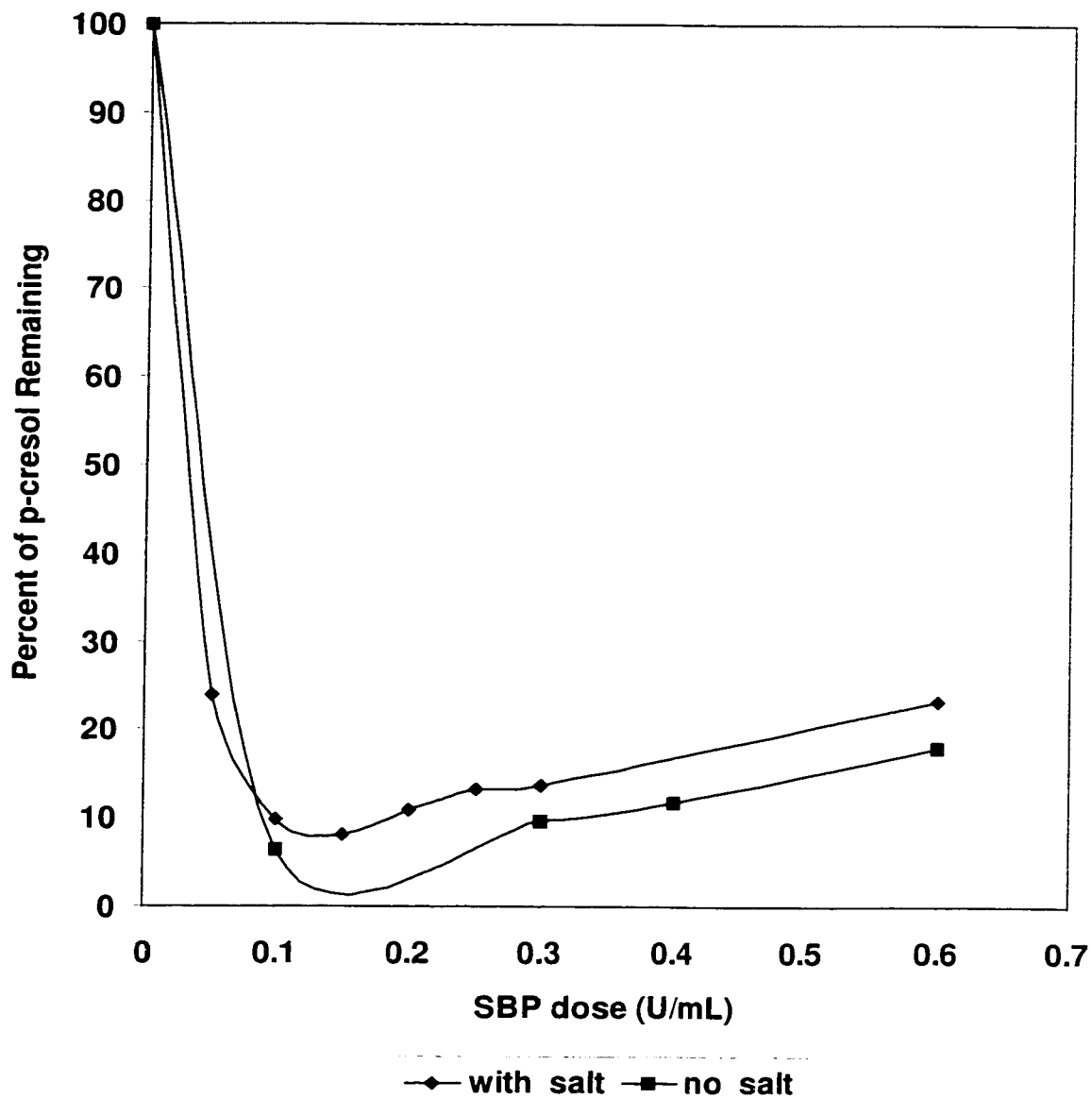
**Reactor Conditions**  
Substrate = 1 mM  
pH = 4.5–5  
PEG = 400 mg/L

**Figure 4B.2.4: Effect of crude SBP (B) dose on the removal of p-cresol**



**Reactor Conditions**  
Substrate = 1mM  
NO PEG  
pH = 4.5–5  
SBP dose = 0.1–0.6 U/mL

**Figure 4B.2.5: Effect of crude SBP (B) dose on the removal of p-cresol**



**Reactor Conditions**  
Substrate = 1 mM  
pH = 4.5–5.0  
NO PEG  
[H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1.6 mM/mM

**Figure 4B.2.6: Effect of crude SBP (B) dose on the removal of p-cresol**

(Figure 4B.2.5). It was found that without PEG, there was a significant improvement in the removal efficiency on increasing the  $\text{H}_2\text{O}_2$  dose. However, the removal efficiency was almost the same for the SBP dose range of 0.1 to 0.6 U/mL. It was then decided to add salt to the solution after the reaction to see if the removal efficiency could be improved due to salt effect (Caza *et al.*, 1999). From Figure 4B.2.6 it is evident that addition of salt did not improve the removal efficiency of the substrate. The optimum enzyme dose was found to be 0.15 U/mL and on increasing the enzyme dose the removal efficiency decreased marginally. This may be due to the increase in  $\text{H}_2\text{O}_2$  demand with an increase in the SBP dose as reported previously in Section 4A.3. Interestingly the effect of higher  $\text{H}_2\text{O}_2$  dose is not observed in the presence of PEG (Figure 4B.2.4). On comparing Figures 4B.2.4 and 4B.2.5 it is observed that PEG had no effect on the removal of p-cresol at  $\text{H}_2\text{O}_2$  dose of 1 mM. However, with higher doses of  $\text{H}_2\text{O}_2$ , PEG had a negative effect on the amount of SBP required for 95% removal of p-cresol. This is inconsistent with the results obtained by Wu. Y. *et al.* (1997) with horseradish peroxidase but is consistent with those obtained by Caza *et al.* (1999) with commercial SBP. Similar to p-cresol, o-cresol also showed little improvement due to the addition of PEG. Both with enzymes A and B, PEG only slightly reduced the amount of SBP required for 95 % removal (Figures 4B.4.1 to 4B.4.3) which is inconsistent with the results obtained by Caza *et al.* (1999) in their experiments using commercial SBP.

The minimum SBP enzyme doses with and without PEG are shown in Table 4B.3. Results obtained from previous experiments using commercial SBP (Caza *et al.*, 1999 and Al-Kassim *et al.*, 1995) indicated higher enzyme requirements for these compounds except p-cresol. The lower enzyme requirements here in these cases could be caused due to usage of crude SBP instead of commercial SBP. The extract of crude SBP contains other unknown constituents, which might affect the enzyme dosage required for cresol removal. The results show that the minimum amount of SBP required for 95 % of removal from initial concentration of the aromatic compounds in the absence of PEG was 1 to 4 times the SBP required with PEG.



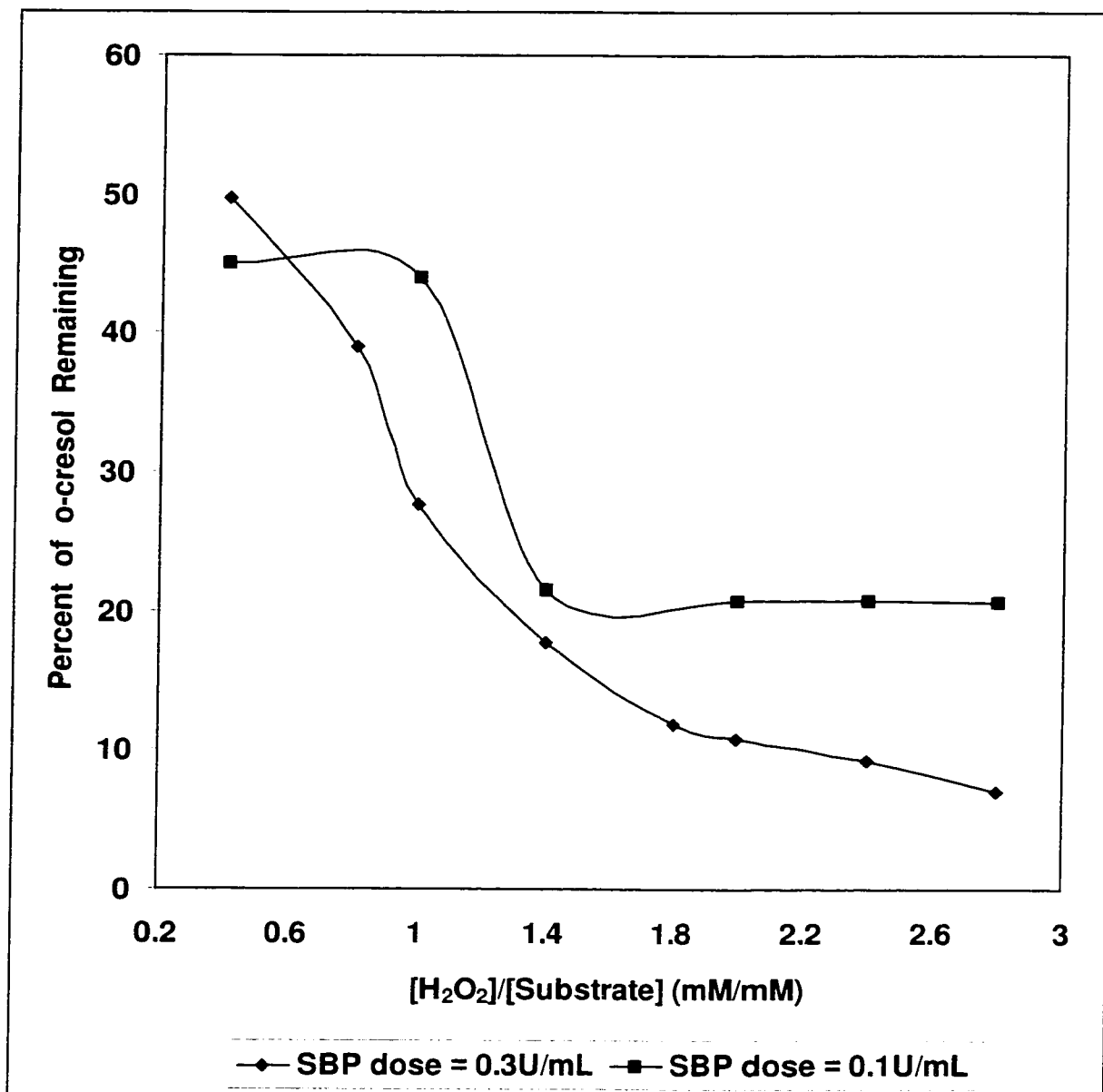
**Table 4B.3 Optimum SBP dose in the presence and absence of PEG.**

| <b>Aromatic compound</b> | <b>Min. SBP dose Without PEG (U/mL)</b> | <b>Min. SBP dose with PEG (U/mL)</b> | <b>SBP dose ratio</b> |
|--------------------------|---|--------------------------------------|-----------------------|
| <b>o-cresol</b>          | <b>0.3 (SBP-A)</b>                      | <b>0.3(SBP-A)</b>                    | <b>1.0</b>            |
| <b>o-cresol</b>          | <b>0.25(SBP-B)</b>                      | <b>0.2(SBP-B)</b>                    | <b>1.25</b>           |
| <b>m-cresol</b>          | <b>0.3(SBP-B)</b>                       | <b>0.075(SBP-B)</b>                  | <b>4.0</b>            |
| <b>p-cresol</b>          | <b>0.15(SBP-B)</b>                      | <b>—</b>                             | <b>—</b>              |

#### **4B.3 Hydrogen Peroxide to Substrate Ratio ( $[H_2O_2]/[Substrate]$ )**

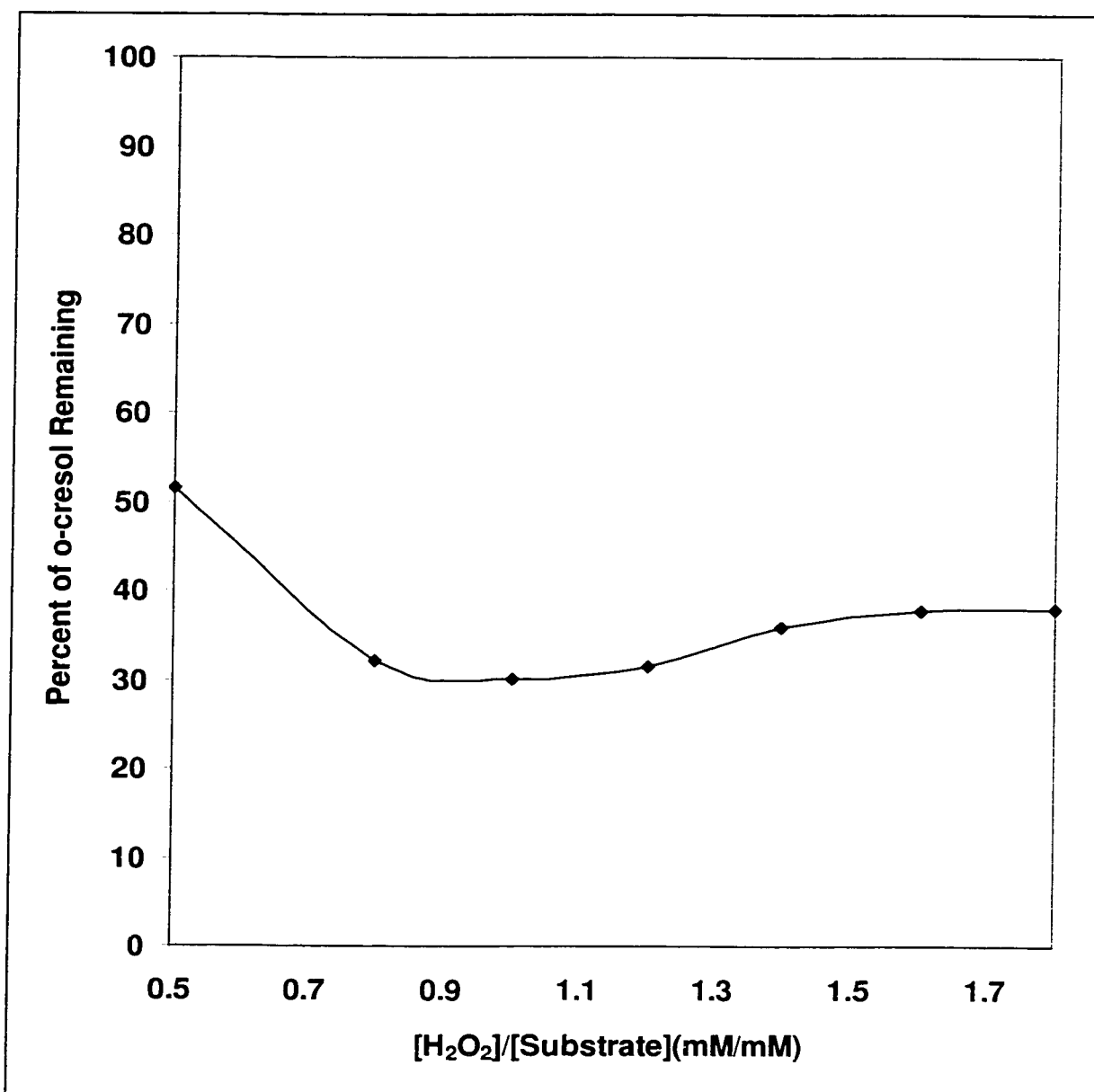
The third parameter that was optimized in this study was the molar ratio of hydrogen peroxide to substrate. These experiments were conducted at the previously determined optimum pH, whereas the initial SBP concentration was kept at less than the optimum dose listed in Table 4B.3 in order to observe a greater change in removal under stressed condition. The PEG dose was kept at an excess dose of 400 mg/L. The concentration for all of the phenolic compounds was kept at 1 mM. Results are plotted in Figures 4B.3.1 to 4B.3.4 and the optimum molar ratios are listed in Table 4B.4.

These figures show that as  $[H_2O_2]/[Substrate]$  was increased the removal efficiency also increased until the optimum value was reached. These results indicate a broad optimum range as was found by Wu. Y. *et al.* (1997) with horseradish peroxidase; however they are inconsistent with those obtained by Caza *et al.*, (1999) when all the compounds showed definite optimum points.



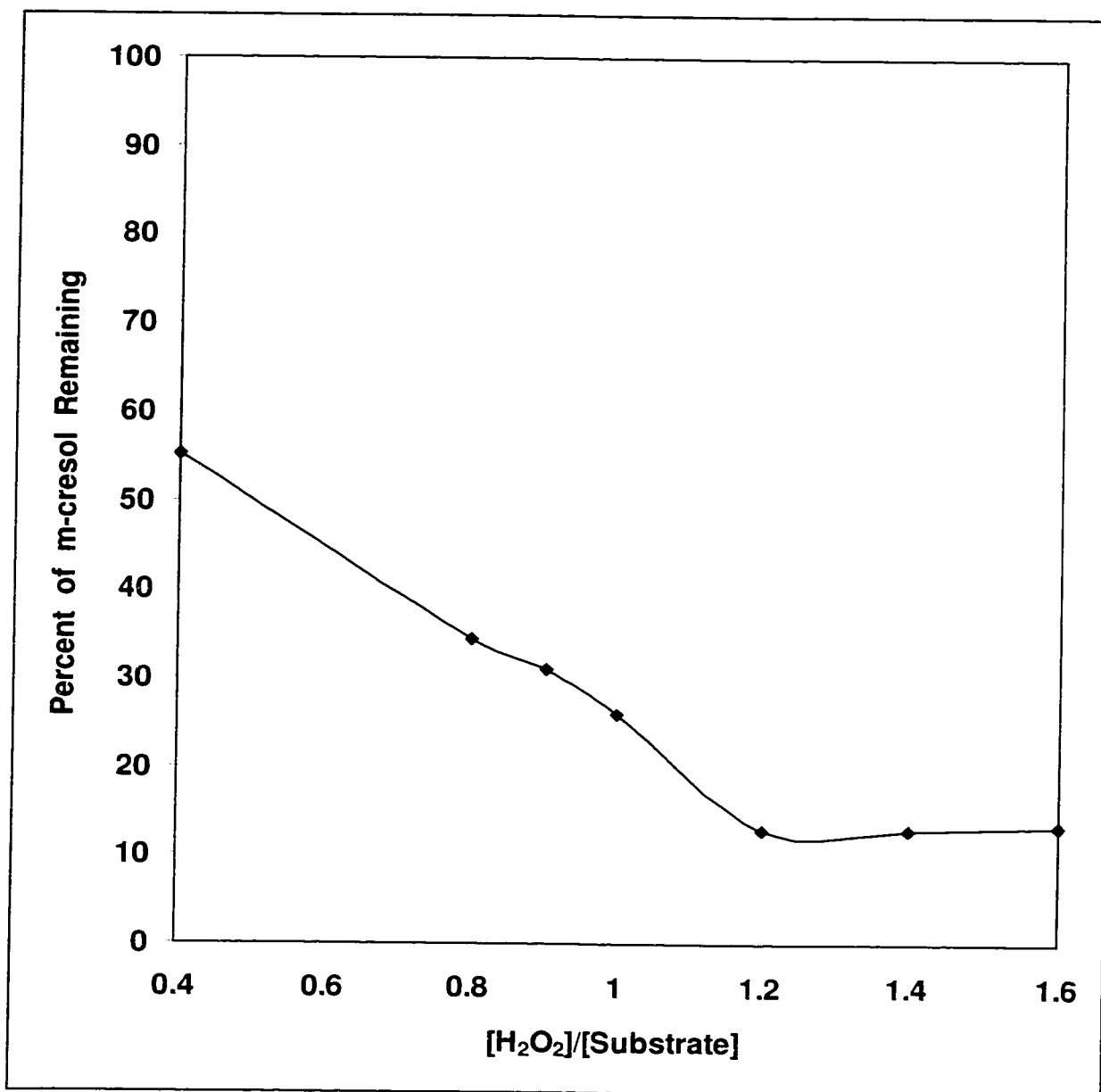
**Reactor Conditions:**  
Substrate = 1mM  
PEG = 400 mg/L  
SBP dose = 0.1–0.3U/mL  
pH = 6–7

**Figure 4B.3.1: Effect of H<sub>2</sub>O<sub>2</sub> dose on the removal of o-cresol using crude SBP (A)**



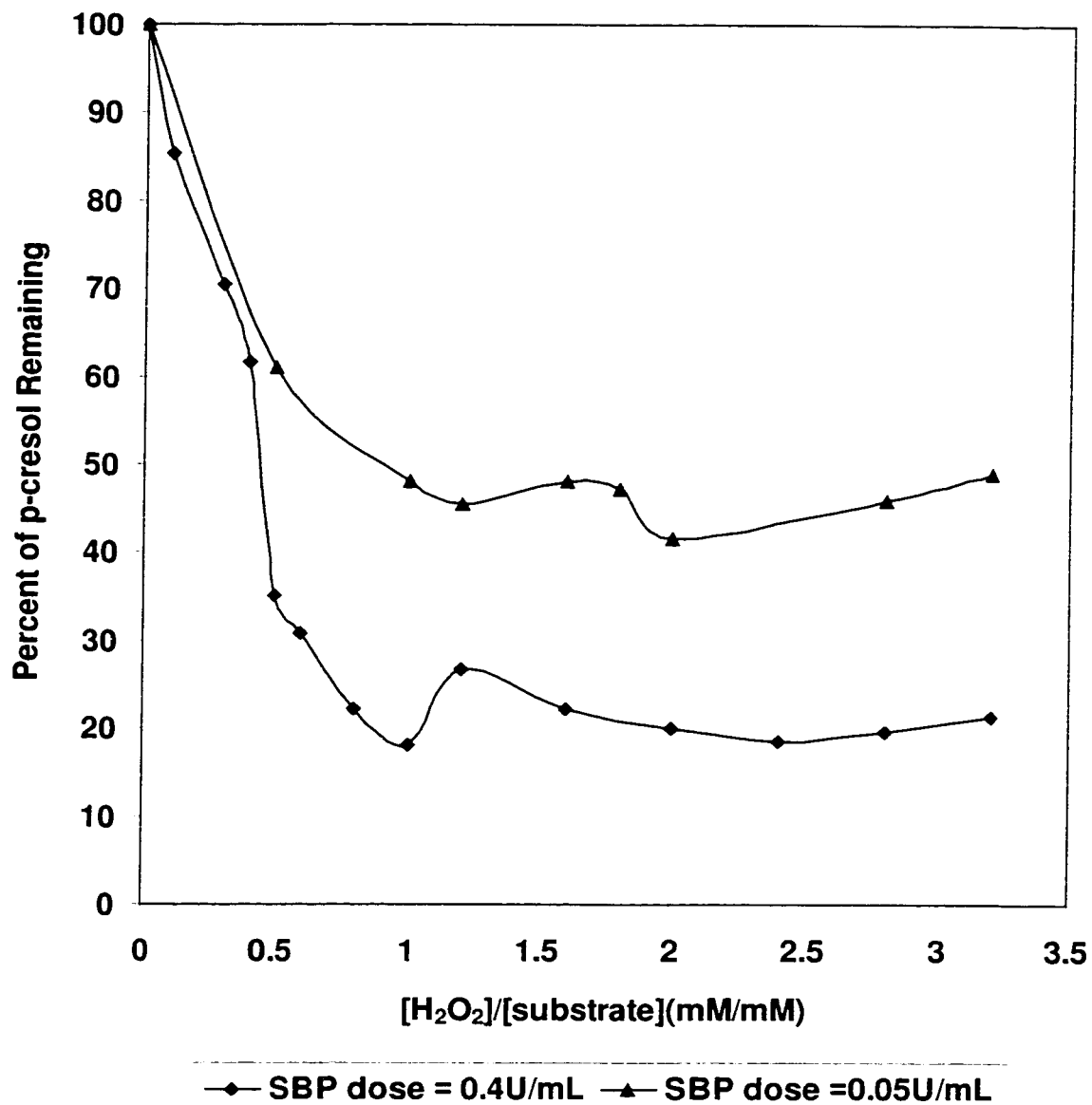
**Reactor Conditions**  
Substrate = 1mM  
SBP dose = 0.1U/mL  
PEG = 400 mg/L  
pH = 6–7

**Figure 4B.3.2: Effect of H<sub>2</sub>O<sub>2</sub> dose on the removal of o-cresol using crude SBP (B)**



**Reactor Conditions:**  
Substrate = 1mM  
PEG = 400 mg/L  
SBP dose = 0.05U/mL  
pH = 5--5.5

**Figure 4B.3.3: Effect of H<sub>2</sub>O<sub>2</sub> dose on the removal of m-Cresol using crude SBP (B)**



**Reactor Conditions:**  
Substrate = 1mM  
PEG = 400 mg/L  
SBP dose = 0.05–0.4U/mL  
pH = 4.5–5

Figure 4B.3.4: Effect of H<sub>2</sub>O<sub>2</sub> dose on the removal of p-Cresol using crude SBP (B)

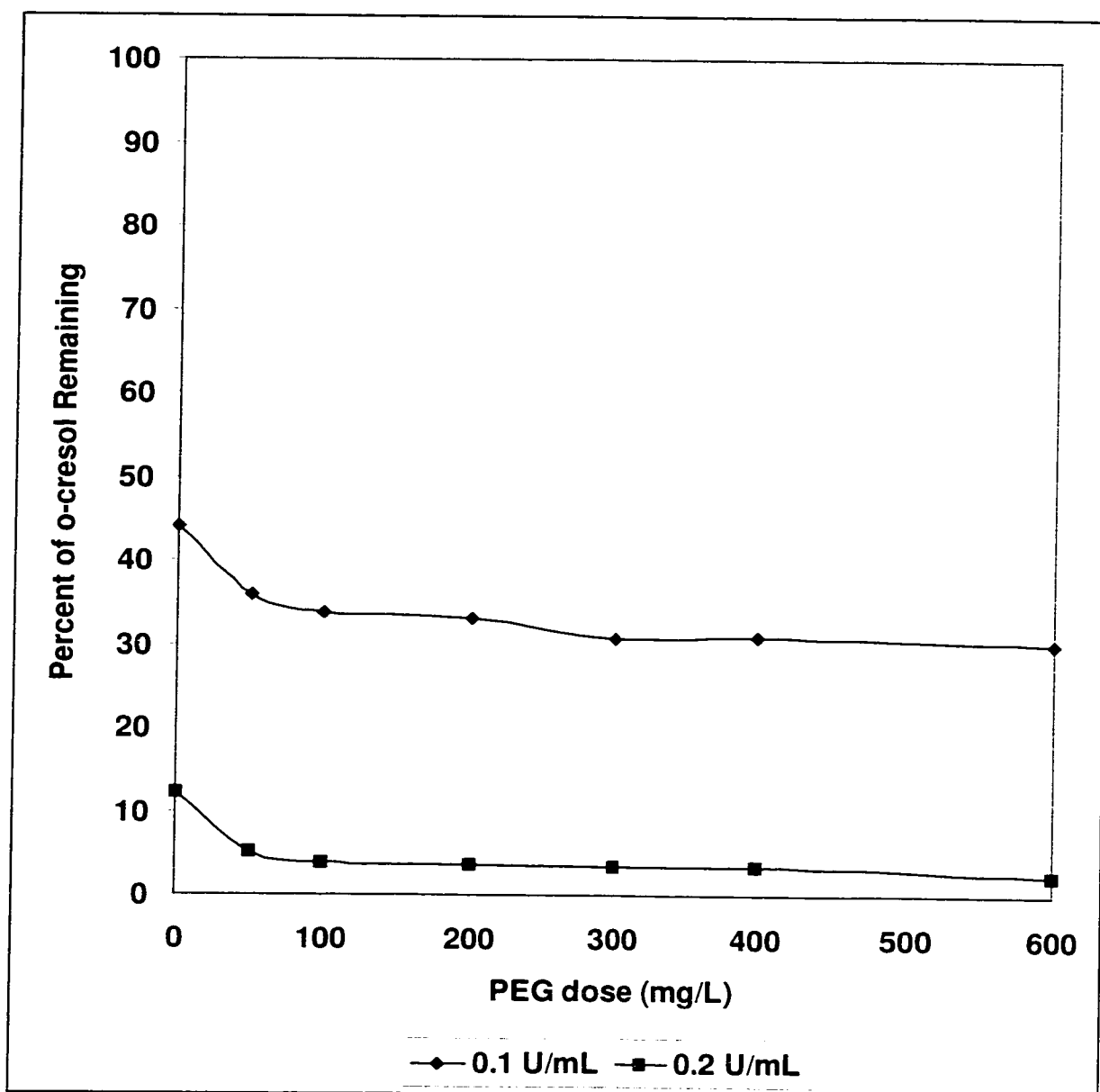
**Table 4B.4: Optimum H<sub>2</sub>O<sub>2</sub> to Substrate ratios for the removal of cresols using crude SBP**

| <b>Aromatic Compound</b> | <b>Type of enzyme</b> | <b>Optimum H<sub>2</sub>O<sub>2</sub> to Substrate Ratio (mM/mM)</b> |
|--------------------------|-----------------------|--|
| <b>o-cresol</b>          | <b>Crude SBP (A)</b>  | <b>1.4</b>   |
| <b>o-cresol</b>          | <b>Crude SBP (B)</b>  | <b>1.0</b>   |
| <b>m-cresol</b>          | <b>Crude SBP (B)</b>  | <b>1.2</b>   |
| <b>p-cresol</b>          | <b>Crude SBP (B)</b>  | <b>1.8</b>   |

As seen in Table 4B.4, the optimum ratios range from 1.0 to as high as 1.8 mM/mM. For the substrate p-cresol, it was observed that without PEG, an increase in [H<sub>2</sub>O<sub>2</sub>]/[Substrate] significantly increased the removal efficiency (Section 4B.2). This was due to high H<sub>2</sub>O<sub>2</sub> demand of the crude SBP as was discussed previously (Section 4A.3). It is possible that when H<sub>2</sub>O<sub>2</sub> is added to the reactor, it may react with the substrate in preference over the crude enzyme. However, the products of reaction will be different for different substrates and their behavior with H<sub>2</sub>O<sub>2</sub> is not known. In the case of p-cresol, the products of reaction might have contributed to the huge amount of H<sub>2</sub>O<sub>2</sub> demand.

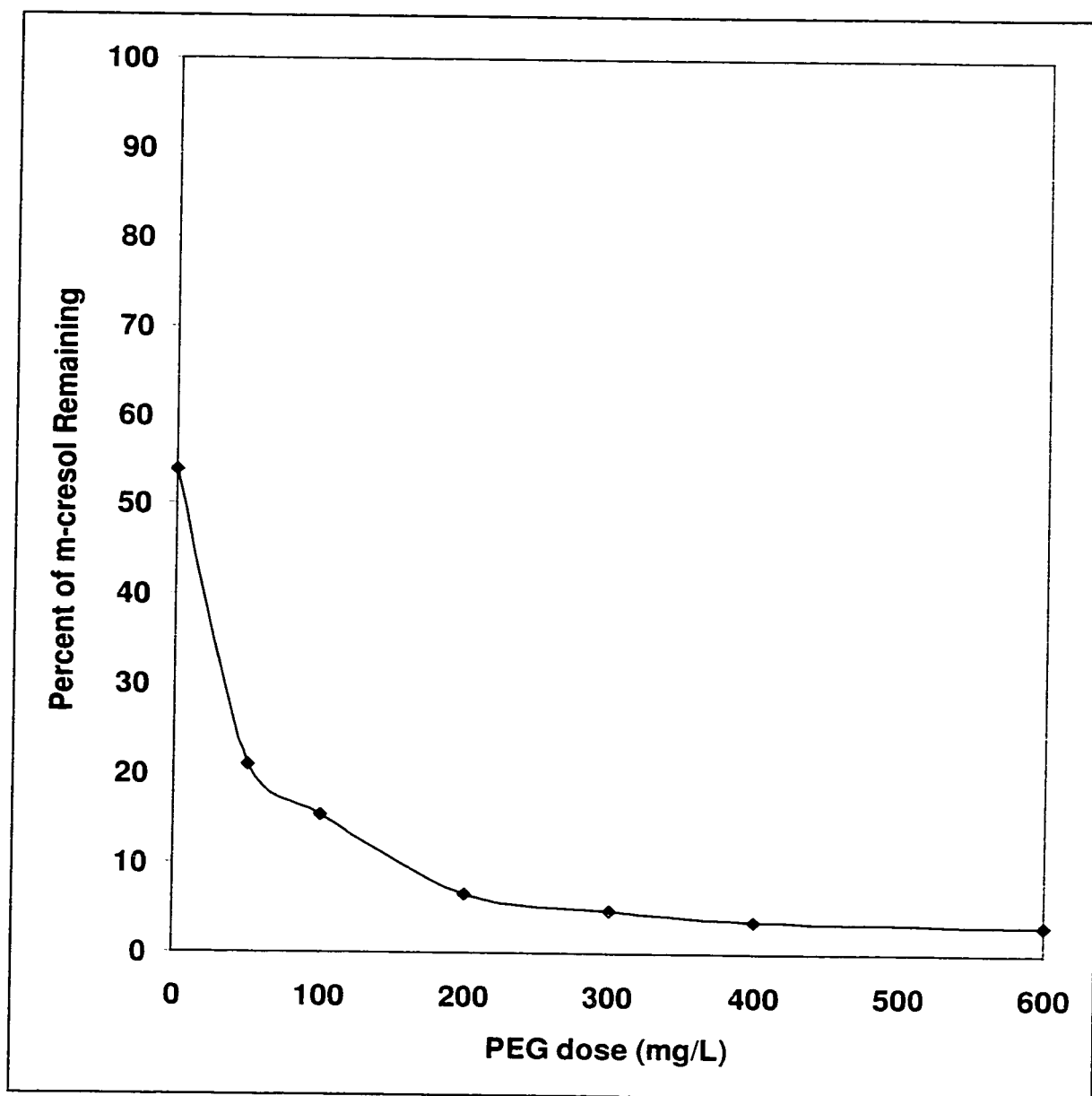
#### **4B.4 Polyethylene Glycol dose**

Polyethylene glycol dose experiments were conducted at the previously established pH and [H<sub>2</sub>O<sub>2</sub>]/[Substrate]. As explained in the previous section, the SBP concentration was kept at less than the optimum. Results for the removal efficiency as a function of PEG dose are plotted in Figures 4B.4.1 through 4B.4.4. The initial concentration of the phenolic compounds was 1 mM for all substrates and the PEG dose ranged from 0 to 600 mg/L.



**Reactor Conditions:**  
Substrate = 1mM  
[H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1 mM/mM  
pH = 6–7

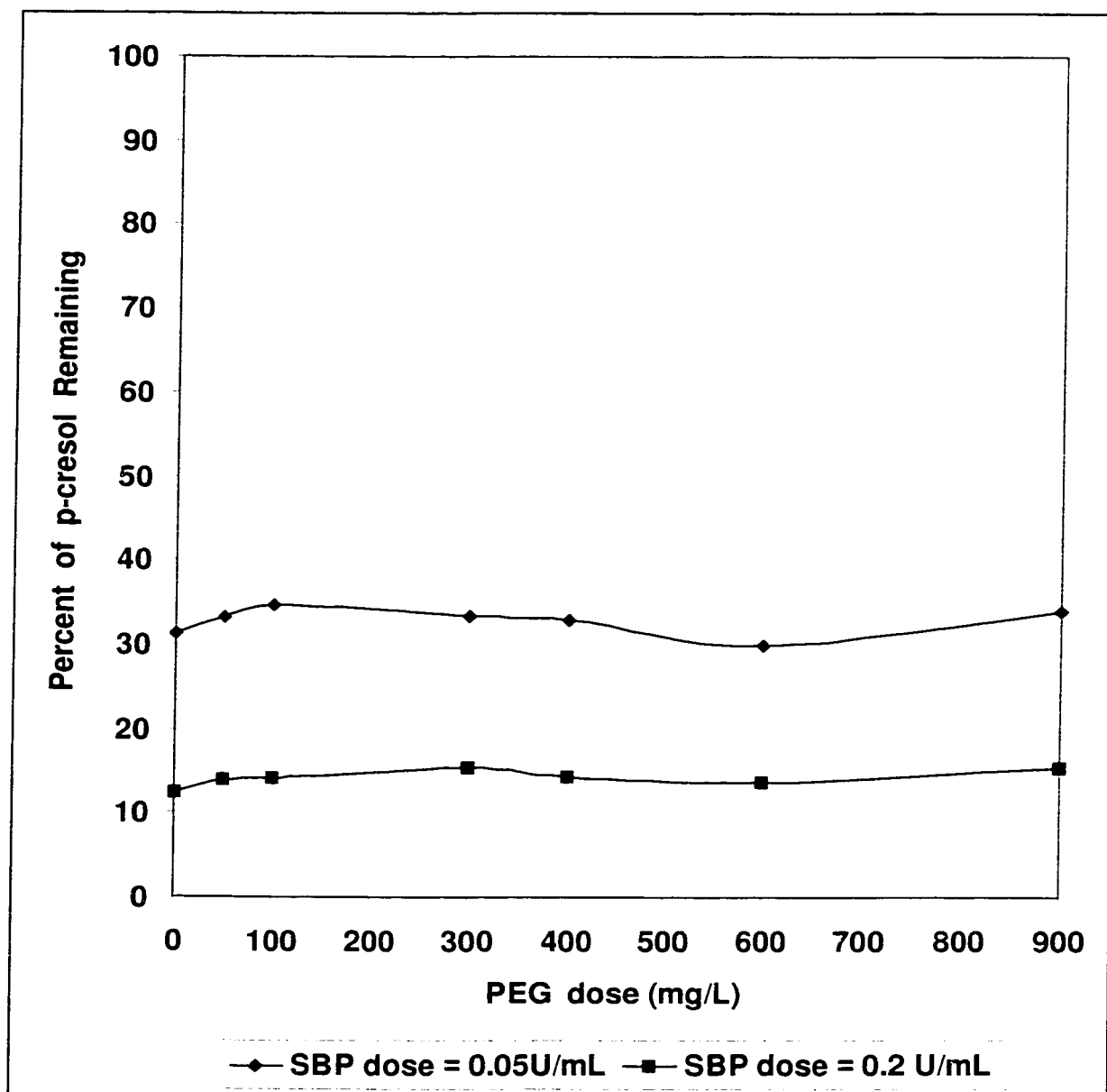
**Figure 4B.4.1: Effect of PEG dose on the removal of o-cresol using crude SBP (B)**



**Reactor Conditions:**  
Substrate = 1 mM  
pH = 5–5.5  
[H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1.2 mM/mM  
SBP dose = 0.1 U/mL

**Figure 4B.4.2: Effect of PEG dose on the removal of m-cresol using crude SBP (B)**





**Reactor Conditions:**  
p-cresol = 1mM  
pH = 4–5  
[H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1mM/mM  
PEG dose = 0–900 mg/L

**Figure 4B.4.3: Effect of PEG dose on the removal of p-cresol using crude SBP (B)**

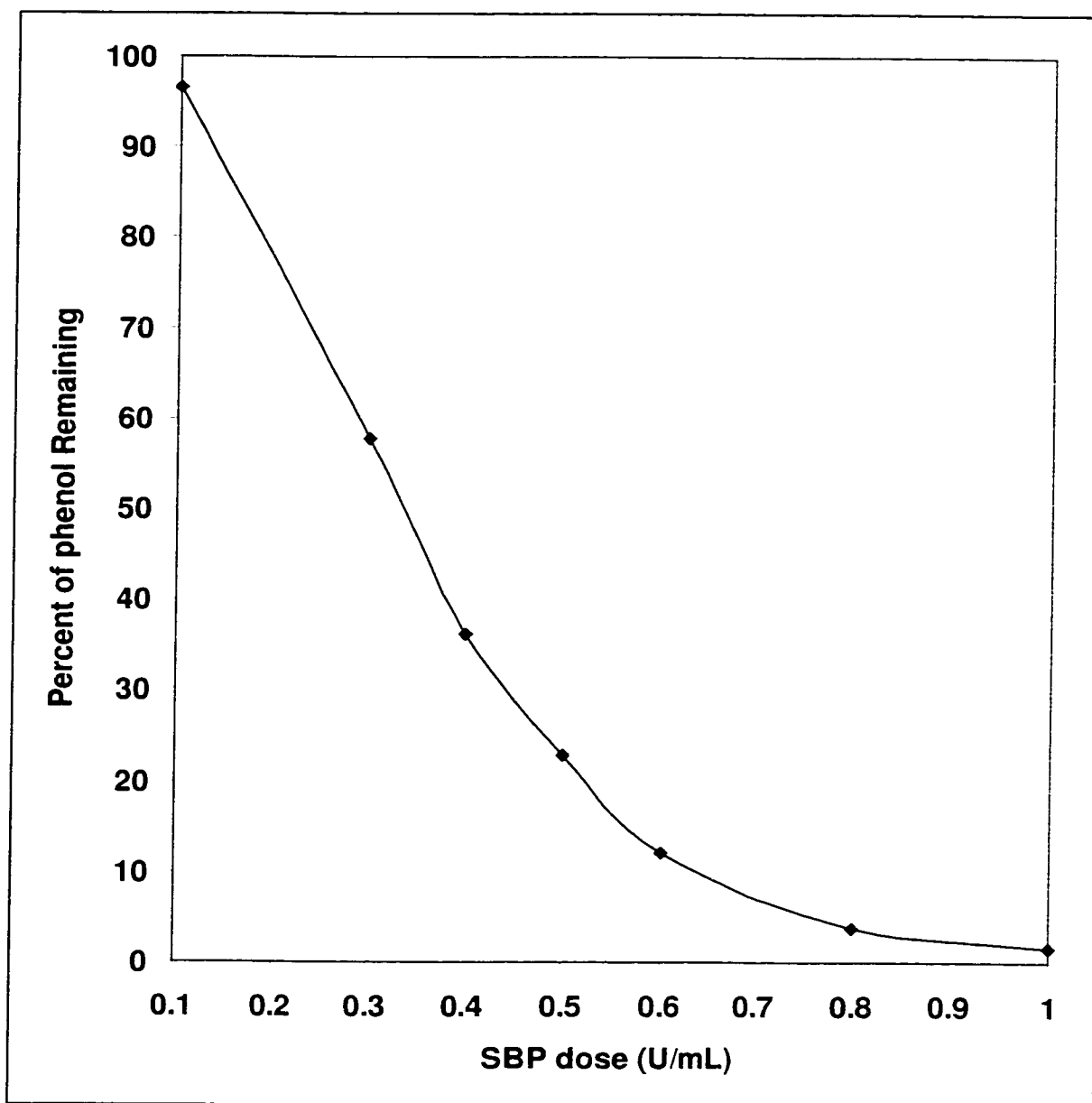
**Table 4B.5: Minimum Effective PEG Dose for removal of cresols using crude SBP**

| <b>Aromatic Compound</b> | <b>Type of enzyme</b> | <b>Minimum Effective PEG Dose (mg/L)</b> |
|--------------------------|-----------------------|--|
| <b>o-cresol</b>          | <b>Crude SBP (B)</b>  | <b>50</b>                                |
| <b>m-cresol</b>          | <b>Crude SBP (B)</b>  | <b>200</b>                               |
| <b>p-cresol</b>          | <b>Crude SBP (B)</b>  | <b>0</b>                                 |

These figures show that, except for m-cresol, the addition of PEG did not improve the removal efficiency. These results differ from those of Caza *et al.*, (1999) and Wu .Y *et al* (1997). This could be due to the fact that the enzyme used was crude SBP instead of commercial SBP or HRP and also due to the fact that these experiments were carried out in un-buffered solutions rather than buffered.

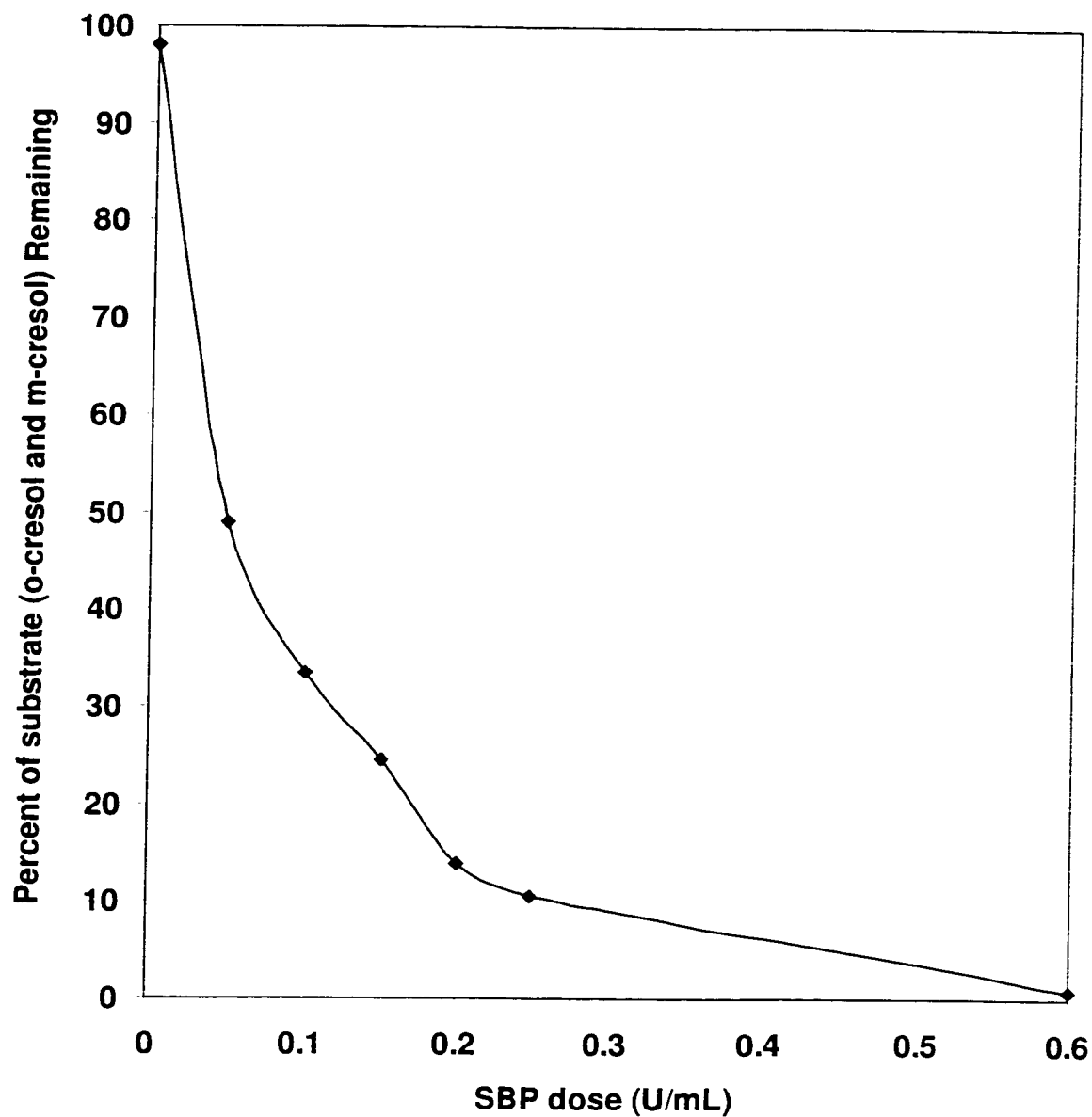
#### **4B.5 Coprecipitation**

The overall removal efficiency of a phenolic compound depends upon its reactivity towards peroxidase and the solubility of the products. Klibanov *et al.* (1980) observed that easily removed substrates often aided in the precipitation of those substrates which were hard to remove. As discussed previously in Section 4B.2, certain compounds required less enzyme than others to achieve a removal efficiency of 95 % or greater. O-cresol, for example, required 0.2 U/mL of SBP, whereas m-cresol required only 0.0175 U/mL to achieve the same removal efficiency. Two sets of experiments were conducted to determine the benefit of coprecipitation on the removal of phenol and o-cresol in the presence of more easily removed compounds. The optimum parameters for the removal of these three compounds individually are listed in Table 4B.6. Except for the optimum SBP dose, the information on other optimum parameters for phenol removal while



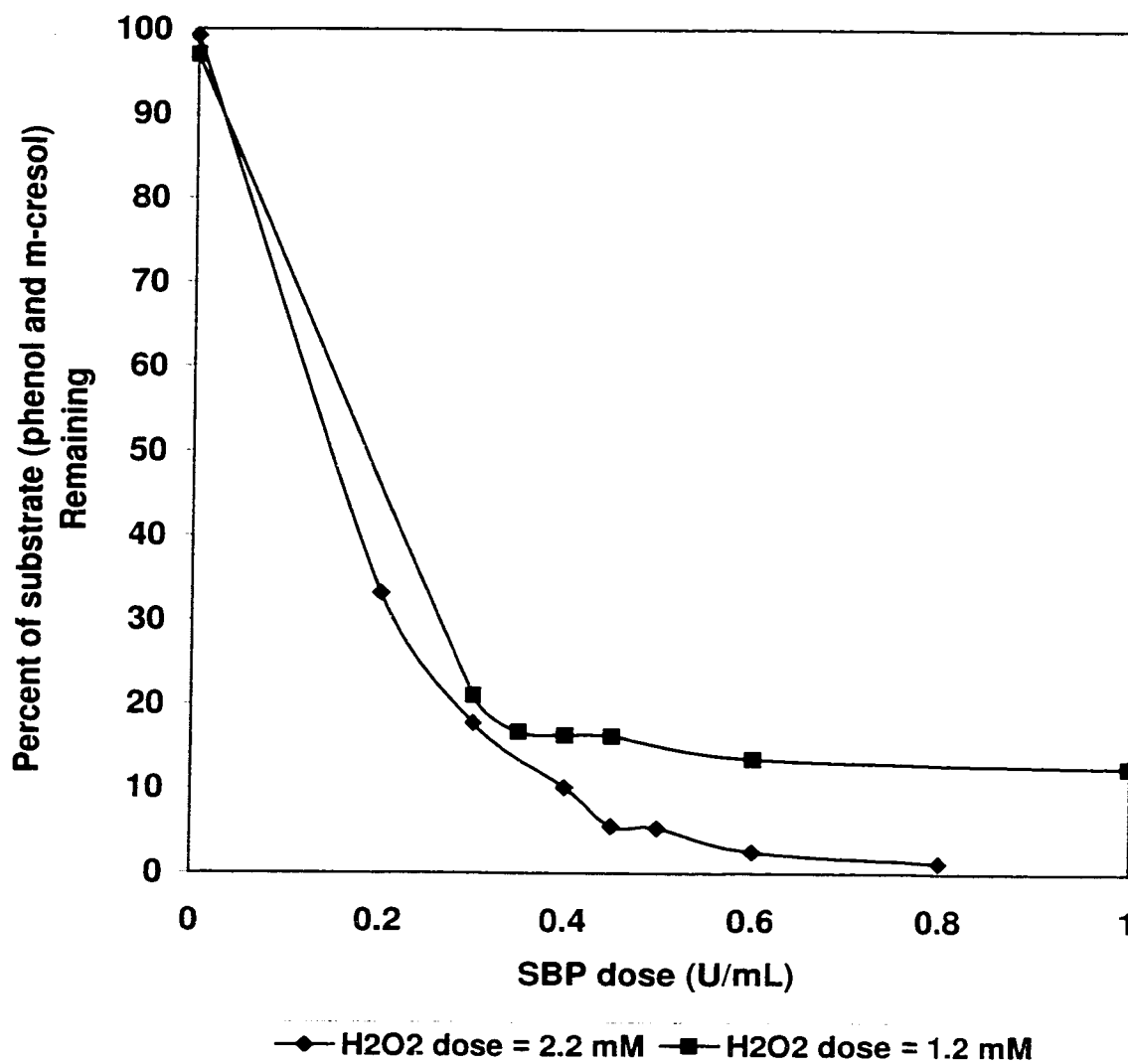
**Reactor Conditions**  
Substrate = 1 mM  
 $[\text{H}_2\text{O}_2]/[\text{Substrate}] = 2.2 \text{ mM/mM}$   
pH = 6  
PEG = 200 mg/L

**Figure 4B.5.1: Effect of crude SBP (B) dose on the removal of phenol**



**Reactor Conditions**  
o-cresol = 0.5 mM  
m-cresol = 0.5 mM  
 $\text{H}_2\text{O}_2$  dose = 1.1 mM  
PEG dose = 200 mg/L  
pH = 5.5–6.0

**Figure 4B.5.2: Effect of crude SBP (B) dose on the removal of two substrates: o-cresol and m-cresol**



**Reactor Conditions**  
Phenol = 0.5 mM  
m-cresol = 0.5 mM  
[H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 2.2 mM  
PEG = 200 mg/L  
pH = 5.9–6.1

**Figure 4B.5.3: Effect of crude SBP (B) dose on the removal of two substrates: phenol and m-cresol**

using crude SBP were obtained by Caza *et al*, 1999. A separate study was conducted to obtain the optimum crude SBP dose for the removal of phenol from synthetic wastewater and the results are shown in Figure 4B.5.1.

**Table 4B.6: Summary of Optimum Parameters for three selected phenolic Compounds**

| <b>Aromatic Compound</b> | <b>SBP dose with PEG (U/mL)</b> | <b>PEG dose (mg/L)</b> | <b>[H<sub>2</sub>O<sub>2</sub>]/[Substrate] (mM/mM)</b> | <b>pH</b>  |
|--------------------------|---------------------------------|------------------------|---|------------|
| <b>Phenol</b>            | <b>0.8</b>                      | <b>50</b>              | <b>1.2</b>  | <b>6</b>   |
| <b>o-cresol</b>          | <b>0.2</b>                      | <b>50</b>              | <b>1.2</b>  | <b>5.5</b> |
| <b>m-cresol</b>          | <b>0.075</b>                    | <b>200</b>             | <b>1.0</b>  | <b>5.5</b> |

In the coprecipitation experiments, the analysis for substrate concentration was done by using the colorimetric method. The amount of individual compound was not determined, but instead, only the total percent of the total substrate remaining was determined. The substrates used for the coprecipitation experiments had almost similar calibration curves and extinction coefficients but not exactly the same. It was assumed to be same and analysis were carried out based on the assumption that both have the same extinction coefficients.

The total initial substrate concentration in coprecipitation experiments was 1 mM with the concentration of each compound being equal to 0.5 mM. Figure 4B.5.2 shows the coprecipitation results for o-cresol and m-cresol. The reaction parameters were as follows: pH = 5.5; PEG dose = 200 mg/L; and [H<sub>2</sub>O<sub>2</sub>]/[Substrate] = 1.1 mM/mM. Table 4B.6 shows that the total enzyme dose required for an individual removal efficiency of 95 % at a concentration of 0.5 mM is 0.14 U/mL (0.1 U/mL for o-cresol plus 0.04 U/mL for m-cresol). From Figure 4B.5.2, the optimum SBP dose is 0.5 U/mL. Therefore the presence of an easier to remove compound (m-cresol) did not aid in the removal of o-cresol.

The results for the removal of phenol in the presence of m-cresol are shown in Figure 4B.5.3. The total initial substrate concentration was 1 mM, with the individual initial concentrations being equal to 0.5 mM. The experiments were conducted at pH = 6,  $[H_2O_2]/[Substrate] = 2.2 \text{ mM/mM}$  and a PEG dose = 200 mg/L. The  $[H_2O_2]/[Substrate]$  ratio used was 2.2 mM/mM instead of 1.2 mM/mM because it was anticipated that by using a hydrogen peroxide dose of 1.2 mM, the peroxidase reaction might run out of hydrogen peroxide due to the high hydrogen peroxide demand of the enzyme (Section 4A.3). This is confirmed by Figure 4B.5.3 where it is found that 95 % removal of substrate could not be obtained even at a high SBP dose of 1.0 U/mL when a hydrogen peroxide dose of 1.2 mM was used. Under similar conditions, the removal of substrate had significantly increased on increasing the hydrogen peroxide dose to 2.2 mM.

The total SBP dose required for 95 % removal in the coprecipitation experiment, calculated from the results in Table 4B.6, is 0.44 U/mL (0.4 U/mL for phenol plus 0.04 U/mL for m-cresol). The optimum SBP dose, from Figure 4B.5.3, was found to be the same. Since there was no improvement in the removal efficiency, it is concluded that the enzymatic removal of phenolic compounds with SBP did not help in coprecipitation. Caza *et al.*, (1999) had arrived at a similar conclusion.

#### **4.6 Source of enzyme**

Experiments for the optimization of the above reaction parameters were conducted with crude SBP enzymes extracted from two different batches/hulls (designated A and B). It was found that the optimum reaction parameters for the removal of cresols from unbuffered tap water were almost same for both SBP extracts A and B. Thus it is evident that irrespective of the crude enzyme source the removal efficiency remains almost same for the removal of cresols from synthetic wastewater.

#### **4.7 Error Analysis**

A set of experiments was conducted in order to determine the reliability of the results in this study. The  $\text{H}_2\text{O}_2$  to substrate ratio series for p-cresol was repeated three times at three different ratios and the percent deviations in results were calculated. Separate solutions were prepared in order to determine the accuracy of analytical techniques. The design parameters were as follows: pH = 4.5; initial p-cresol concentration = 1.0 mM; PEG dose = 0 mg/L; and SBP enzyme dose = 0.4 U/mL. The results of these experiments are listed in Table 4B.7.

These results indicate that the deviation was always less than  $\pm 5\%$ , which is within an acceptable range. Therefore, the results obtained in this study are considered to be accurate and reliable.



**Table 4B.7: Error Analysis**

| <b>Sample #</b> | <b>[H<sub>2</sub>O<sub>2</sub>]/[Substrate]<br/>(mM/mM)</b> | <b>Percent<br/>Remaining</b> | <b>Average</b>   |
|-----------------|---|------------------------------|------------------|
| <b>1</b>        | <b>0.4</b>  | <b>60.2</b>                  | <b>60 ± 0.21</b> |
| <b>2</b>        | <b>0.4</b>  | <b>60.1</b>                  |                  |
| <b>3</b>        | <b>0.4</b>  | <b>59.8</b>                  |                  |
| <b>4</b>        | <b>0.8</b>  | <b>19.5</b>                  | <b>20 ± 0.64</b> |
| <b>5</b>        | <b>0.8</b>  | <b>20.4</b>                  |                  |
| <b>6</b>        | <b>0.8</b>  | <b>21.1</b>                  |                  |
| <b>7</b>        | <b>1.6</b>  | <b>23</b>                    | <b>22 ± 1.1</b>  |
| <b>8</b>        | <b>1.6</b>  | <b>22</b>                    |                  |
| <b>9</b>        | <b>1.6</b>  | <b>20.9</b>                  |                  |

## **5. CONCLUSIONS AND RECOMMENDATIONS**

### **5.1 Conclusions**

A suitable experimental protocol has been developed for extracting crude SBP enzyme from soybean hulls and to optimize hulls to solvent ratio and time of extraction. Also, pertinent characteristic properties of the crude enzyme have been determined. These included temperature stability, hydrogen peroxide demand and phenol content in the enzyme.

#### **5.1.1 Extraction of crude enzyme from hulls**

- The optimum time of extraction was found to be less than 40 minutes.
- The hulls to solvent ratio had no optimum value. The total units of enzyme extracted remained constant and did not depend on the volume of the solvent added to soybean hulls. Thus, about 50 and 80 activity units of enzyme were obtained from 1 g of soybean hulls (A) and soybean hulls (B) respectively. Depending upon the strength of enzyme required, the hulls to solvent ratio can be selected.
- Tap water as a solvent was found to be more suitable than buffer solution. Irrespective of the amount of solvent added to 10 g of soybean hulls, about 50 mL of the extracted solution was not recovered.
- The crude SBP was found to be extremely stable up to 80<sup>0</sup>C. On the other hand, commercial SBP was unstable above 50<sup>0</sup>C. Thus, the crude SBP enzyme has better potential for applications at high temperatures.
- The crude SBP showed a significant hydrogen peroxide demand. This demand depends on the pH of the enzyme solution and also on the activity of the enzyme. The hydrogen peroxide demand was found to be maximum at pH 6—7. It increased with an increase in the enzyme activity. The information on the hydrogen peroxide demand of crude SBP under given conditions helps in determining the hydrogen peroxide dose required for peroxidase reactions during removal of substrates.

- The phenol content in the crude SBP was found to be very small as compared to substrate concentration and it did not interfere with the analysis of the phenolic compounds during the enzymatic treatment of synthetic wastewater. It was also found that the phenolic content in the crude SBP was directly proportional to the activity of the enzyme.

### **5.1.2 Application of crude SBP for cresol removal**

- The results have demonstrated the feasibility of using crude soybean peroxidase (SBP) enzyme for treating cresols. The reaction parameters that were optimized to achieve a removal efficiency of at least 95 % were pH, SBP enzyme dose with and without PEG,  $[H_2O_2]/[Substrate]$  and PEG dose. Table 5.1 summarizes the optimum doses for all of these reaction parameters.
- The optimum pH varied between 4.5 and 6.5 for different substrates. Both m-cresol and p-cresol had a narrow optimum pH range.
- Excess peroxidase had no significant effect on the removal of cresols; however, limiting the amount of SBP resulted in lower substrate removal efficiencies. The addition of PEG did not reduce the amount of enzyme required for 95 % removal, except for m-cresol in which case it reduced by 4 times.
- The hydrogen peroxide requirement depended on the enzyme dose because of the high demand of hydrogen peroxide by the crude SBP. The optimum  $H_2O_2$  to substrate molar ratios ranged from 1.0 for o-cresol to 1.8 for p-cresol. As the molar ratio increased, the removal efficiency also increased until the optimum ratio was achieved. Beyond the optimum ratio, there was no change in the removal efficiency.
- The addition of PEG had little effect on improving the substrate removal efficiency, except for m-cresol in which case the removal efficiency increased due to PEG. An excess of PEG had no measurable effect on the removal efficiency, except for p-cresol, in which case excess PEG drastically decreased the removal efficiency.

**Table 5.1: Summary of Optimum Reaction Parameters for Various Aromatic Compounds**

| <b>Aromatic Compound</b> | <b>Type of enzyme</b> | <b>Optimum pH</b> | <b>Optimum SBP dose without PEG (U/mL)</b> | <b>Optimum SBP dose with PEG (U/mL)</b> | <b>[H<sub>2</sub>O<sub>2</sub>]/[Substrate] (mM/mM)</b> | <b>Minimum Effective PEG dose (mg/L)</b> |
|--------------------------|-----------------------|-------------------|--|---|---|--|
| <b>o-cresol</b>          | Crude SBP (A)         | 6.5               | 0.30                                       | 0.30                                    | 1.4   |  |
| <b>o-cresol</b>          | Crude SBP (B)         | 6.0               | 0.25                                       | 0.20                                    | 1.0   | 50                                       |
| <b>m-cresol</b>          | Crude SBP (B)         | 5.5               | 0.30                                       | 0.075                                   | 1.2   | 200                                      |
| <b>p-cresol</b>          | Crude SBP (B)         | 4.5               | 0.15                                       | --                                      | 1.8   | 0  |
| <b>Phenol</b>            | Crude SBP (B)         | 6*                | --   | 0.8                                     | 2.2   | 200*                                     |

\* Values taken from Caza *et al.*, 1999

- Coprecipitation of phenol and o-cresol with an easier to remove compound did not reduce the SBP requirements for 95 % removal. In the presence of m-cresol, the enzyme requirements for the removal of phenol and o-cresol did not improve. Similarly, the minimum SBP dose for phenol in the presence of m-cresol was not reduced significantly.

## 5.2 Recommendations

The results of these experiments have shown that crude soybean peroxidase is a viable alternative to other enzymes that have previously been studied. In order to implement the enzymatic method of treatment to full-scale industrial applications, several other aspects must be considered.

The potential toxicity of the final products must be studied. Once the nature of the by-products is determined, a suitable disposal method can be chosen. After extraction, the enzyme hulls could then be used as animal feed. This would greatly reduce the amount of solid waste that is produced and in turn would also reduce costs. Similarly some researchers have also suggested using crude HRP (Cooper *et al.*, 1996; Dec *et al.*, 1994; Klibanov, 1982) to reduce treatment costs. In contrast to crude SBP, crude HRP produces a greater amount of solid waste. Another advantage of using soybeans is that they are more readily available than horseradishes. A detailed cost analysis should be carried out in order to determine the applicability of this process over current treatment methods.

The effect of co-precipitation should continue to be investigated. Co-precipitation may yield better results in a mixture of compounds other than the ones used in this study. Other components that are present in an actual wastewater stream may interfere with or improve the removal of substrates.

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**APPENDIX A**  
**Enzyme Activity Assay**

## 1. General

The purpose of the enzyme activity assay is to determine the amount of active enzyme that is present in a solution. The reaction between phenol and  $\text{H}_2\text{O}_2$  is catalyzed by the enzyme (SBP) such that the products of the reaction react with AAP to form red coloured solution which absorbs light at a peak wavelength of 510 nm. Under saturated conditions of phenol, AAP and  $\text{H}_2\text{O}_2$ , the initial rate is measured by observing the change in colour formation in a solution.

## 2. Reagents

- i) Phosphate buffer (0.1 M NaPP, pH 7.4)
  - 160 mL of 0.2 M monobasic sodium phosphate +
  - 840 mL of 0.2 M dibasic sodium phosphate +
  - distilled water to make 2 L
  
- ii) Phenol (0.1 M phenol)
  - 1882.2 mg phenol +
  - Phosphate buffer to make 200 mL +
  - Store in refrigerator
  
- iii) 4-Aminoantipyrine (9.6 mM AAP)
  - 390 mg AAP make +
  - Phosphate buffer to 200 mL.
  - Store in refrigerator
  
- iv) Hydrogen peroxide (2.0 mM  $\text{H}_2\text{O}_2$ )
  - a) 226.7  $\mu\text{L}$  of 30 %  $\text{H}_2\text{O}_2$  +
  - distilled water to make 100mL
  - b) 10 mL of  $\text{H}_2\text{O}_2$  solution from (a) +
  - distilled water to make 100 mL

### 3. Procedure

In a semi-micro cuvette, combine in the following order:

50  $\mu\text{L}$  SBP solution

500  $\mu\text{L}$  NaPP buffer

100  $\mu\text{L}$  0.1 M phenol

250  $\mu\text{L}$  9.6 mM AAP

100  $\mu\text{L}$  2.0 mM  $\text{H}_2\text{O}_2$

The total volume in the cuvette should be 1 mL, and the rate of colour formation must be measured before substrate depletion becomes significant. Immediately after the addition of the sample, shake the cuvette and then monitor the absorbance change with time at 510 nm.

### 4. Calculation

i) Find the average slope over the linear range of the data in terms of absorbance units per unit time (au/min).

ii) Calculate the activity in the cuvette

$$\text{Activity}(U / mL) = \frac{\text{slope}(au / \text{min})}{6000 \text{ au} \cdot L / \text{mol}} \times 10^6 \frac{\mu\text{mol}}{\text{mol}} \times \frac{1(L)}{1000(mL)}$$

The activity is given in terms of micromoles of hydrogen peroxide converted per minute at 20°C and pH 7.4.

iii) Calculate the activity of the sample

$$\text{Activity in sample}(U / mL) = \text{Activity in cuvette}(U / mL) \times \frac{1000(\mu\text{L})}{\text{sample volume}(\mu\text{L})}$$

**APPENDIX B**  
**Aromatic Substrate Assay**

## 1. General

This is a colorimetric assay used to measure the concentration of an aromatic substrate in an aqueous sample. It uses ferricyanide and 4-aminoantipyrine as colour generating substances when combined with the aromatic compound in the sample. The limiting reagent is the amount of the aromatic compound, and therefore, the degree of colour generated at a peak wavelength of 510 nm is proportional to the amount of aromatic present.

## 2. Reagents

- i) Ferricyanide reagent (83.4 mM of  $\text{K}_3\text{Fe}(\text{CN})_6$  in 0.25 M  $\text{NaHCO}_3$ )  
2.75 g  $\text{K}_3\text{Fe}(\text{CN})_6$  +  
2.1 g  $\text{NaHCO}_3$  +  
distilled water to make 100 mL.
- ii) 4-Aminoantipyrine reagent (20.8 mM of AAP in 0.25 M  $\text{NaHCO}_3$ )  
0.423 g AAP +  
2.1 g  $\text{NaHCO}_3$  +  
Distilled water to make 100 mL

## 3. Procedure

In a semi-micro cuvette, combine in the following order:

800  $\mu\text{L}$  of aromatic sample (diluted if necessary)

100  $\mu\text{L}$  of AAP reagent

100  $\mu\text{L}$  of ferricyanide reagent

The final assay sample volume should be 1 mL. After a couple of minutes, measure the absorbance at 510 nm against a reagent blank.

## 4. Calculation

Using the appropriate calibration curve (Appendix D), convert absorbance readings into desired concentration units.

**Appendix-C**  
**Hydrogen peroxide assay**

## 1. General

This end-point colorimetric assay was used to measure the concentration of hydrogen peroxide in a sample. The assay uses *Arthromyces ramosus* peroxidase as a catalyst and 4-aminoantipyrine as a color generating cosubstrate in combination with an aromatic substrate, phenol, in the assay mixture. In this assay, the amount of hydrogen peroxide introduced into the assay sample is the only limiting reactant; therefore, the degree of the colour developed in the reaction is proportional to the amount of peroxide in the sample. Once the maximum amount of colour has developed, the absorbance (at 510 nm) is converted to the hydrogen peroxide concentration in the cuvette by means of a calibration curve. The hydrogen peroxide concentration in the sample is then calculated according to the dilution the sample had undergone in the cuvette.

## 2. Preparation of reagents

- a) Phosphate buffer (0.5M NaPP, pH 7.4)

In a 1000 mL flask, add the following

13.796 g of monobasic sodium phosphate ( $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ),

56.78 g of dibasic sodium phosphate ( $\text{Na}_2\text{HPO}_4$ ),

add distilled water up to 1L.

- b) Phenol (0.1M Phenol) in 0.5M phosphate buffer pH 7.4

Dissolve 9.411 g of phenol on 1000 mL of 0.5M phosphate buffer solution.

- c) Assay mixture

In a beaker, add the following:

41 mg of 4-aminoantipyrine (AAP),

10 mL of 0.1M phenol in 0.5M Phosphate buffer pH 7.4,

200  $\mu\text{L}$  of ARP stock solution,

9.8 mL distilled water,

The final total volume of the assay reagent is 20 mL.

### **3. Calibration Procedure**

Make up a stock solution of hydrogen peroxide with a concentration of 1.0 mM. From this stock solution prepare standards ranging from 0 to 1.0 mM. In a test tube place the following solutions

200  $\mu\text{L}$  of the assay reagent

750  $\mu\text{L}$  distilled water

50  $\mu\text{L}$  of standard sample

The total volume of the assay mixture must be 1 mL and hydrogen peroxide concentration in the assay mixture should be below 50  $\mu\text{M}$ . Immediately after the addition of hydrogen peroxide standard, shake the tube and then wait until the colour is fully developed (mostly after 10 minutes). Put the assay mixture in a semi-micro cuvette and read the maximum amount of absorbance at 510 nm. Repeat the procedure for all standards, using triplicate measurements. Make a plot of absorbance versus hydrogen peroxide concentration in the cuvette, and determine the slope of the trace using linear regression. A typical calibration curve for the hydrogen peroxide is presented in Figure A-5.

### **4. Measurement of hydrogen peroxide**

In a semi-micro cuvette, place these reactants in the following order:

200  $\mu\text{L}$  of the assay reagent,

0 to 750  $\mu\text{L}$  distilled water,

50 to 800  $\mu\text{L}$  of sample.

The total volume in the cuvette must be 1 mL. Immediately after addition of the sample, shake the cuvette and then wait for the full development of the colour. Read the maximum amount of absorbance at the peak wavelength of 510 nm. Determine the cuvette  $\text{H}_2\text{O}_2$  concentration from the calibration curve.



## 5. Calculations

Calculate the sample hydrogen peroxide concentration from:

$$[H_2O_2]_{Sample} = [H_2O_2]_{Cuvette} \times \left( \frac{1000 \mu L}{Sample Volume \mu L} \right)$$

## **Appendix-D**

### **Standard Curves for the Aromatic Compounds**

The following section consists of the standard curves (Beer's fit) for the aromatic compounds and  $\text{H}_2\text{O}_2$ .

According to Beer's law,

$$A = \epsilon C L$$

where  $A$  = Absorbance

$\epsilon$  = Molar extinction coefficient ( $\text{M}^{-1}.\text{cm}^{-1}$ )

$C$  = Concentration (M)

$L$  = Length of the path of light traveled in cm (1 cm in these cases)

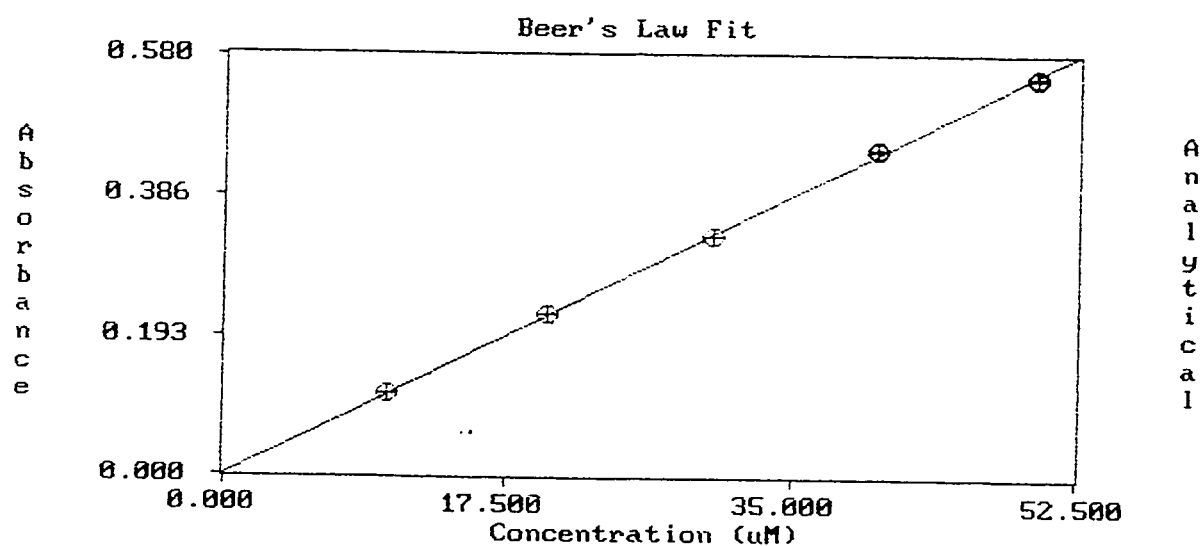
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Date : 04-30-1999  
Time : 11:48:08  
Operator : Not Entered

File Name : C:\NUV\DATA\KBOCR-CO.STD

Sample Name : o-cresol  
Solvent Name : tap water  
Conc Units :  $\mu\text{M}$

Analytical Wavelength : 506 nm  
Reference Wavelength : None Selected  
Confirmation Wavelengths : None Selected  
Integration Time : 1 seconds



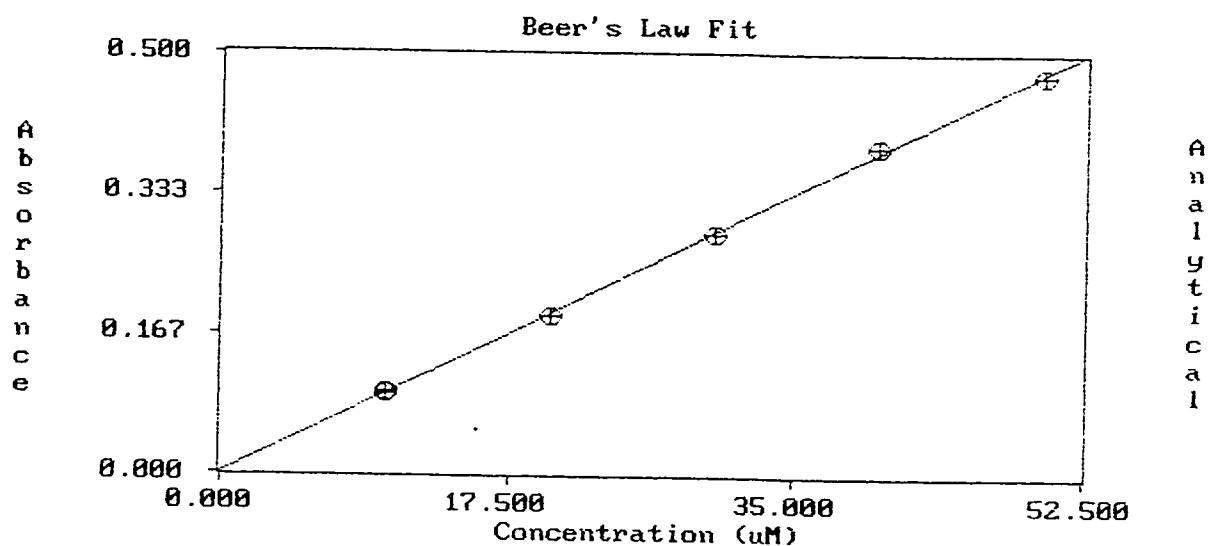
## ---&gt; Standard Calibration Report &lt;---

Date : 04-30-1999  
Time : 11:45:55  
Operator : Not Entered

File Name : C:\UV\DATA\KBMCR-00.STD

Sample Name : m-cresol  
Solvent Name : tw(boiled)  
Conc Units :  $\mu\text{M}$

Analytical Wavelength : 502 nm  
Reference Wavelength : None Selected  
Confirmation Wavelengths : None Selected  
Integration Time : 1 seconds



## ---&gt; Standard Calibration Report &lt;---

Date : 04-30-1999

Time : 11:53:34

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File Name : C:\UV\DATA\KBPCR-CO.STD

Sample Name : p-cresol

Solvent Name : NaOH (0.1M)

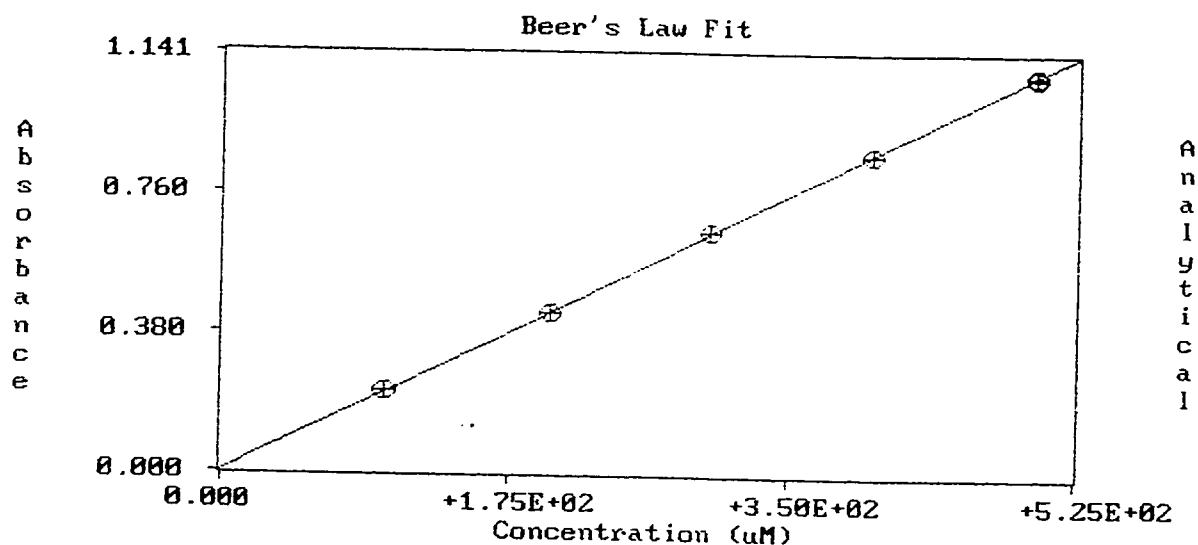
Conc Units :  $\mu\text{M}$ 

Analytical Wavelength : 296 nm

Reference Wavelength : None Selected

Confirmation Wavelengths : None Selected

Integration Time : 1 seconds



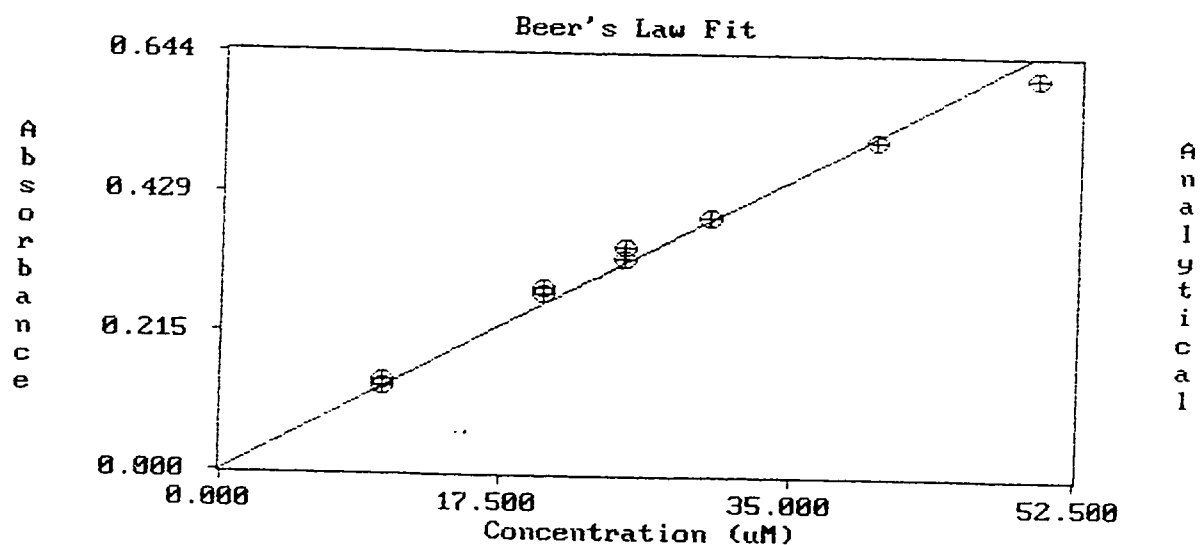
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Date : 04-30-1999  
Time : 11:43:54  
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File Name : C:\UV\DATA\KBPHCA.STD

Sample Name : PHENOL  
Solvent Name : TAP WATER  
Conc Units :  $\mu\text{M}$

Analytical Wavelength : 510 nm  
Reference Wavelength : None Selected  
Confirmation Wavelengths : None Selected  
Integration Time : 1 seconds



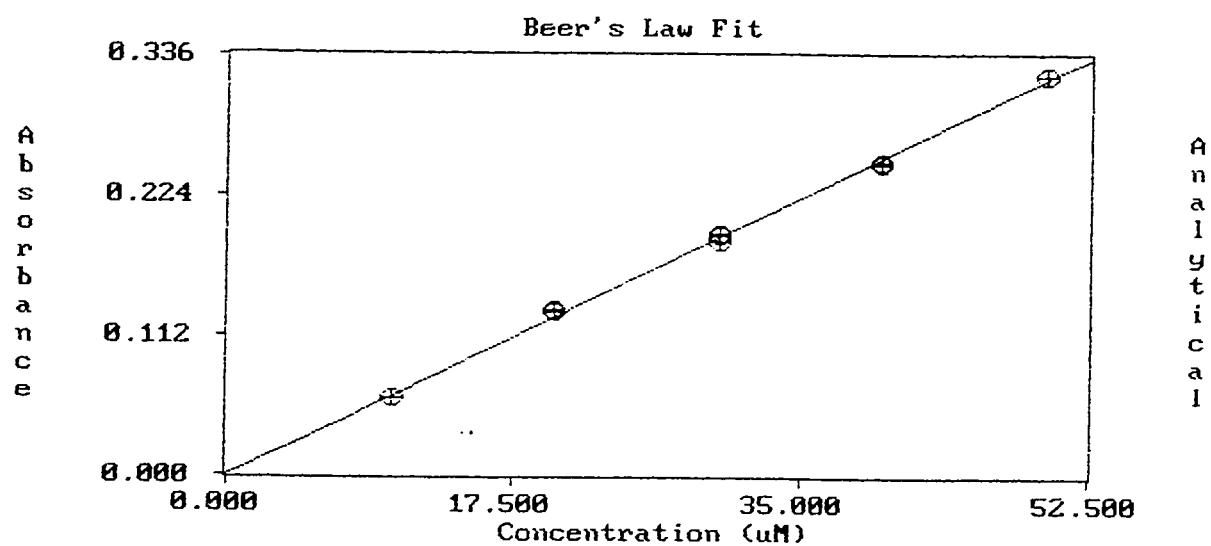
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Time : 11:42:02  
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Sample Name : H2O2  
Solvent Name : TAP WATER  
Conc Units :  $\mu\text{M}$

Analytical Wavelength : 510 nm  
Reference Wavelength : None Selected  
Confirmation Wavelengths : None Selected  
Integration Time : 1 seconds



## VITA AUCTORIS

**Name:** Kaushik Biswas

**Place of birth:** Calcutta, India

**Date of birth:** September 25<sup>th</sup>, 1973

**Education:** Master of Applied Science  
Environmental Engineering  
University of Windsor  
Windsor, Ontario, Canada  
1998-1999

Bachelor of Technology  
Chemical Engineering  
Indian Institute of Technology  
Kharagpur, India  
1993-1997

**Work Experience:** Graduate Assistant, Research Assistant  
Civil and Environmental Engineering  
University of Windsor  
Windsor, Ontario, Canada  
January 1998- June 1999

Scientist Engineer  
National Council for Cements and Building Materials  
Ballabgarh, Haryana, India  
May-December, 1997

Vocational Trainee  
Indian Oil Corporation  
Barauni, India  
May- July, 1996

**Awards:** Summer Research Scholarship, 1999  
Visa Differential fee waiver, 1998-1999